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IN THE CIRCUIT COURT OF THE 11TH  
JUDICIAL CIRCUIT IN AND FOR DADE  
COUNTY, FLORIDA

GENERAL JURISDICTION DIVISION

CASE NO.: 94-08273 CA(20)

FBN: 614009

HOWARD A. ENGLE, M.D., et al

Plaintiffs

VS

RJ REYNOLDS TOBACCO COMPANY,  
et al.,

Defendants

DEPOSITION

OF

DR. JAMES GIANNINI

DEPOSITION taken before me, Lisa C. Nagy-Baker, a  
Notary Public within and for the State of Ohio, on the 24th  
Day of June, A.D., 1998, pursuant to Notice and at the time  
and place therein specified, to be used pursuant to the  
Rules of Civil Procedure or by agreement of counsel in the  
above cause of action, pending in the Court of Common  
Pleas, within and for the County of Dade, State of Florida.

\*REC'd disk

\*REC'd condensed

\*REC'd exhibits

**COPY**

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2 JUDICIAL CIRCUIT IN AND FOR DADE  
3 COUNTY, FLORIDA  
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18 above cause of action, pending in the Court of Common  
19 Pleas, within and for the County of Dade, State of Florida.  
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25

1 APPEARANCES  
2  
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Shook, Hardy & Bacon, LLP  
\*Rec'd desk  
\*Rec'd full  
\*Rec'd Exhibits

1 3  
2  
3 STIPULATIONS *99: case manager*  
4  
5 It is stipulated and agreed by and between *Engl.*  
6 counsel for the parties hereto that the deposition may be *can.*  
7 taken at this time, 1:30 p.m., June 24, 1998, at 871 McKay  
8 Court, Boardman, Ohio. *Rec'd*  
9 It is further stipulated and agreed by and  
10 between counsel that the deposition may be taken in  
11 shorthand by Lisa C. Nagy-Baker, a Notary Public within and  
12 for the State of Ohio, and may be by her transcribed with  
13 the use of computer-assisted transcription; that the  
14 witness will read and sign the finished transcript of his  
15 deposition.  
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## STIPULATIONS

It is stipulated and agreed by and between counsel for the parties hereto that the deposition may be taken at this time, 1:30 p.m., June 24, 1998, at 871 McKay Court, Boardman, Ohio.

It is further stipulated and agreed by and between counsel that the deposition may be taken in shorthand by Lisa C. Nagy-Baker, a Notary Public within and for the State of Ohio, and may be by her transcribed with the use of computer-assisted transcription; that the witness will read and sign the finished transcript of his deposition.

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DEFENDANT'S EXHIBITS INTRODUCED: NONE



1 WHEREUPON,  
2 JAMES GIANNINI, M.D.,  
3 of lawful age, being by me first duly  
4 sworn to testify the truth, the whole  
5 truth, and nothing but the truth, as  
6 hereinafter certified, deposes and  
7 says as follows:

8 CROSS EXAMINATION:

9 BY MR. HOAG

10 Q Can you state your name for the record,  
11 please.

12 A My name is James Giannini.

13 Q And you have a medical degree; is that  
14 correct, sir?

15 A That is correct.

16 Q And what year did you graduate from  
17 medical school?

18 A 1974.

19 Q What medical school did you go to?

20 A The University of Pittsburgh.

21 Q And at what point when you were at the  
22 University of Pittsburgh at the medical school did you  
23 decide to specialize in psychiatry?

24 A Somewhere between my junior and senior  
25 year.

1 Q That would have been somewhere after you  
2 had completed two years of medical school?

3 A That is correct.

4 Q So those first two years of medical  
5 school were not, you obviously were not specializing in  
6 psychiatry; correct?

7 A Well, no one can specialize in the first  
8 two years of medical school.

9 Q Right. That's exactly right. And I just  
10 wanted to get that on the record. And those first two  
11 years of medical school, what courses did you take?

12 A My freshman year I took gross anatomy,  
13 histology, embryology, genetics, biochemistry,  
14 neurosciences, a course called medical  
15 psychology/psychiatry; there may have been others, but  
16 those were the major courses. Oh, yes, microbiology.

17 Q During those first two years, did the  
18 subject of cigarette smoking ever get mentioned by any of  
19 the medical school professors?

20 A It might have.

21 Q Do you recollect whether it did or not?

22 A No, I cannot at this time.

23 Q Was disease causation any part of the  
24 curriculum in medical school?

25 A Yes.

1 Q And what part of the curriculum was that?

2 A That was included in morbid anatomy or  
3 pathology, same course. It was included in -- are we still  
4 talking about the first two years?

5 Q Yes.

6 A It was included in Physical Diagnosis and  
7 Principles of Medicine.

8 Q And you took all those courses?

9 A And I wasn't finished, and in pediatrics.

10 Q And you took these courses?

11 A That is correct.

12 Q Did you pass all these courses?

13 A Yes, I did.

14 Q Did Surgeon Generals' reports ever get  
15 mentioned during any of these courses?

16 A Not that I can recall.

17 Q Have you ever had occasion to read or  
18 review any report of the United States Surgeon General?

19 A Yes.

20 Q Which report or reports have you read or  
21 reviewed?

22 A '64 and '88 recently and, in the distant  
23 past, I believe there was one in '77.

24 Q Now, the 1964 Surgeon General's report  
25 was about lung cancer and smoking; correct?

1 A It was about smoking, period.

2 Q And that report concluded that cigarette  
3 smoking causes lung cancer; correct?

4 A To the best of my knowledge, my  
5 recollection, I limited my perusal to the effects on the  
6 central nervous system.

7 Q You limited your perusal of the 1964  
8 Surgeon General's report to the effects on the central  
9 nervous system?

10 A That is correct.

11 Q Have you ever reviewed any of the  
12 epidemiological studies related to cigarette smoking and  
13 health?

14 A As it relates to the central nervous  
15 system.

16 Q You never reviewed any epidemiological  
17 studies other than those that relate directly to the  
18 central nervous system; is that correct?

19 A That is correct.

20 Q And during the third year of medical  
21 school, did you take any courses other than psychiatric  
22 courses or courses in the field of psychiatry?

23 A In the third year, yes.

24 Q What courses did you take that year?

25 A Internal medicine, research. Research

1 was second and third year. Internal medicine, psychiatry,  
2 general surgery, neurosurgery, anesthesiology, obstetrics  
3 and gynecology.

4 Q And what did you do in the fourth year of  
5 medical school; what did you take or do?

6 A Which question are you asking me?

7 Q What do you mean, which question?

8 A You asked me what I took, and you asked  
9 me what I did.

10 Q Well, let's break it down. What courses  
11 did you take the fourth year?

12 A I took advanced internal medicine, child  
13 psychiatry, psychiatric research, advanced adult  
14 psychiatry, three courses in community psychiatry. There  
15 may have been one other course that I can't recall at the  
16 time.

17 Q What was the curriculum for advanced  
18 internal medicine?

19 A In advanced internal medicine, I was  
20 assigned to a professor and we comprised the unit which  
21 consisted of junior medical students, a single senior  
22 medical student, myself, who functioned as the senior  
23 student and had some supervisory and teaching  
24 responsibilities, an intern, a resident, a faculty member;  
25 and then there was another senior medical student whose

1 function was to investigate certain particular disease  
2 states; and he functioned as a partial intern.

3 Q What is internal medicine?

4 A Internal medicine is the study and  
5 treatment of disease in adults.

6 Q Did it include the study and treatment of  
7 lung cancer?

8 A It did.

9 Q And during that course, did the subject  
10 of cigarette smoking ever come up?

11 A Not to my recollection.

12 Q During the entire time that you were in  
13 medical school, did the relationship between cigarette  
14 smoking and any disease ever get discussed, to your  
15 recollection?

16 A It may have. I cannot recollect  
17 specifically.

18 Q After you completed medical school, did  
19 you have any opinion concerning whether or not cigarette  
20 smoking caused any disease?

21 A No.

22 Q After you completed medical school, did  
23 you have any opinion as to whether or not cigarette smoking  
24 had ever resulted in the premature death of at least one  
25 human being who smoked cigarettes?

1 MR. KEMNA: Objection. I'm going to  
2 object to this continuing line of questioning on causation  
3 issues. Dr. Giannini has not been offered as an expert to  
4 discuss causation between cigarette smoking and disease;  
5 and it's fairly clear on his expert disclosure statement,  
6 so just note that objection.

7 Q Go ahead. You can answer.

8 A Could you please repeat the question.

9 MR. HOAG: Could the court reporter  
10 repeat the question, please.

11 (Whereupon the record was read as requested.)

12 A Not that I can recall.

13 Q As you sit there today while you're  
14 taking this deposition by telephone with me asking  
15 questions by telephone, do you have any opinion as to  
16 whether or not cigarette smoking causes any disease?

17 MR. KEMNA: Objection. Dr. Giannini  
18 is not being offered for the purpose of expressing opinions  
19 on the causation issue. He is not being offered as an  
20 expert on causation.

21 MR. HOAG: For purposes of this  
22 deposition, I'm just asking him this question and I want to  
23 know the answer. We are allowed to cross-examine witnesses  
24 as to their knowledge, their expertise and their  
25 credibility as witnesses; and I'm just asking him as a

1 medical doctor as he sits there today if he has an opinion  
2 as to whether or not cigarette smoking causes any disease.

3 MR. KEMNA: And I'll repeat my  
4 objection.

5 MR. HOAG: We've already heard it.  
6 It's preserved.

7 Q Now, would you please answer the  
8 question.

9 Could the court reporter repeat the question, please?  
10 (Whereupon the record was read as requested.)

11 A My answer would be I've read that  
12 cigarette smoking may be a risk factor, but I lack  
13 expertise in this area to assess these evaluations.

14 Q Do you consider risk factor and cause to  
15 be the same thing?

16 A No.

17 Q Is it more likely than not, Doctor, that  
18 cigarette smoking has resulted in the premature death of at  
19 least one human being who smoked cigarettes in the United  
20 States of America?

21 MR. KEMNA: Objection. Dr. Giannini  
22 is not offered as an expert on causation issues.

23 Q You can answer.

24 A Okay. Since I lack expertise in this  
25 area, I'm unable to answer.



1 Q Are you saying that you do not have  
2 enough expertise as a medical doctor to know whether or not  
3 it is more likely than not that cigarette smoking has  
4 resulted in the premature death of at least one human being  
5 in the United States of America?

6 MR. KEMNA: Objection. Same  
7 objection as before.

8 A When we're talking more likely, that's  
9 hardly scientific. More likely than not. Secondly, I'm  
10 not an expert in epidemiology or internal medicine.

11 Q So what is your answer to the question;  
12 you don't know?

13 A My answer is I lack sufficient expertise  
14 to give a response as an expert.

15 Q Well, I'm not even asking you whether or  
16 not you consider yourself to be an expert. I just asked  
17 you as you sit here today with the education you have,  
18 whatever that may be, and with the knowledge you have,  
19 whatever that may be, is it more likely than not that  
20 cigarette smoking has resulted in the premature death of at  
21 least one human being who smoked cigarettes in the United  
22 States of America?

23 MR. KEMNA: Objection. Dr. Giannini  
24 is not being offered as an expert on the issue, and this  
25 question has been asked and answered.

1 MR. HOAG: It hasn't been answered  
2 yet.

3 Q Go ahead, answer.

4 A My answer is I lack expertise to respond  
5 to your inquiry.

6 Q Have you ever published any research on  
7 the effects of nicotine?

8 A Yes.

9 Q Can you point to me on your CV where  
10 those are?

11 A I don't have my CV.

12 MR. HOAG: Does the defense counsel  
13 have a copy of his curriculum vitae?

14 MR. KEMNA: John, I intended to bring  
15 one with me, but unfortunately I must have left it at the  
16 office. I don't have a copy with me today.

17 MR. HOAG: Okay. I'll just fax it  
18 over.

19 MR. KEMNA: Fax it where?

20 MR. HOAG: Do you guys have a fax  
21 machine where you are?

22 MR. KEMNA: I don't know if they have  
23 a fax machine. This is a conference center in Boardman,  
24 Ohio, and we have the facilities of a speaker phone; but I  
25 have no idea whether they have a fax machine available.

1 MR. HOAG: Do you want to give me  
2 their phone number? I'll call and ask if you don't want to  
3 bother.

4 MR. KEMNA: Hold on a second. Let's  
5 take a break for five minutes or so, and I'll see if we can  
6 locate somebody who can give an answer to that.

7 (Whereupon a brief recess was taken.)

8 MR. KEMNA: John, I located somebody  
9 here; they said they have a fax number. They'll try to  
10 pass the document through to us as soon as it arrives. The  
11 number is 330-726-3894.

12 MR. HOAG: Whose attention should I  
13 make it for?

14 MR. KEMNA: Do it to my attention,  
15 Don Kemna.

16 (Whereupon the record was read as requested.)

17 Q The research you've done on the effects  
18 of nicotine, has it been published in a peer review  
19 journal?

20 A Yes.

21 Q When did you do this research?

22 A The early 1980s.

23 Q And did you do more than one -- did you  
24 publish more than one article on the effects of nicotine?

25 A I only published one.

1 Q One article. What was the title of the  
2 article?

3 A I can't recall.

4 Q Do you remember what you concluded in  
5 that article, if anything?

6 A Yes. That Clonidine reduced symptoms of  
7 discontinuation of nicotine or of cigarette smoking, to be  
8 more accurate.

9 Q Clonidine reduced what; I'm sorry?

10 A Discontinuation, or discontinuance  
11 symptoms.

12 Q And when you used the word discontinuance  
13 of symptoms, what are you talking about?

14 A When smoking was discontinued.

15 Q What are the discontinuance symptoms of  
16 discontinuing smoking?

17 A In my paper?

18 Q Yeah, what did you write in your paper?

19 A I can't recall. I'd need to look at my  
20 paper.

21 Q At least in your paper you considered  
22 there to have been discontinuance symptoms to stopping  
23 smoking; is that correct?

24 A In my paper I considered it worth  
25 investigating.

1 Q You even said that Clonidine reduced  
2 those symptoms; is that correct?

3 A That's what I concluded.

4 Q What is Clonidine?

5 A Clonidine is an anti-hypertensive.

6 Q And what does that mean in layperson's  
7 terms?

8 A It's used to treat high blood pressure.

9 Q And did you actually prescribe or observe  
10 the behavior of individuals who were attempting to quit  
11 smoking who had been given Clonidine?

12 A I supervised. I did not do it directly.  
13 It was a blinded study.

14 Q What do you mean, blinded study?

15 A The people who observed did not know who  
16 was getting Clonidine and who was not.

17 Q And were the people that were trying to  
18 quit smoking in both of the groups?

19 A I'd have to look at the paper.

20 Q You don't remember now as you're sitting  
21 there today whether or not both of the groups of people  
22 were people who were trying to quit smoking?

23 A I can't remember if I had a control group  
24 or not.

25 Q Well, was the group of people you were

1 looking at people who were trying to quit smoking?

2 A Yes. I didn't know if they were trying  
3 to quit. We had them quit.

4 Q What do you mean, you had them quit?

5 A That was a condition of being in the  
6 study.

7 Q A condition of being in the study was  
8 that at least during the length of the study they had to  
9 stop smoking cigarettes?

10 A That is correct.

11 Q And all the people that were in this  
12 study were prior to that time regular cigarette smokers; is  
13 that correct?

14 A I can't recall.

15 Q Well, was it necessary for them to be  
16 regular cigarette smokers in the study for the study to be  
17 a valid study?

18 A See, regular is rather an imprecise term.  
19 There were criteria for length of time and the amount. I  
20 can't recall 16 years later.

21 Q And what led you to the conclusion in the  
22 paper that it reduced discontinuance symptoms, the  
23 Clonidine?

24 A What led me is we used some sort of  
25 rating scale. The raters, as I can recall, subjectively

1 gave them some sort of number on the rating scale. We  
2 added up the numbers; and the numbers, depending on the  
3 rating scale, were either lower or higher.

4 Q So the rating scale was subjective rather  
5 than objective; is that right?

6 A It was subjective in the sense that the  
7 raters had to observe certain phenomena.

8 Q What phenomena did they have to observe?

9 A It would depend on what rating scale we  
10 used.

11 Q What rating scale did you use?

12 A I would have to review the paper.

13 Q Were the individuals who stopped smoking  
14 during the course of this study asked any questions?

15 A I don't understand. What kind of  
16 questions?

17 Q I don't know. Any kind of questions.  
18 Were they asked any questions as part of this study in  
19 order to collect the data?

20 A Yes.

21 Q What questions were they asked?

22 A Our normal procedure asked their name,  
23 their address, if they're on any medication, their gender,  
24 their predominant race, minority race. We usually ask  
25 weight and age.

1 Q And the responses that you measured, or  
2 the symptoms that you measured, were they measured based on  
3 what the person told you; or were they measured based on  
4 physiological measurements that were not at all based on  
5 what the patient was saying or the subject was saying?

6 A To answer your question, it's actually  
7 two responses. I know we were not measuring symptoms per  
8 se. We were measuring phenomena. Secondly, it was not  
9 objective. There were no -- we did not use instruments to  
10 measure objectively physiological change. It was all  
11 subjective.

12 Q In other words, it was all based on what  
13 the subjects were telling you about how they felt; is that  
14 right?

15 A It was either what the subjects were  
16 telling us, what my associates observed, or some  
17 combination thereof, depending on the rating scale used.

18 Q So the associates were actually measuring  
19 some observable change in the behavior of the subjects?

20 A Possibly. Again, see, I don't remember  
21 which rating scale I employed.

22 Q And what journal was this article  
23 published in?

24 A Society for Neuroscience Abstracts.

25 Q And you said you only did one article on



1 nicotine, and you did that in the early 1980s. Why did you  
2 not follow-up and do additional studies on nicotine?

3 A I did.

4 Q Oh, you only published one article,  
5 though?

6 A That's correct.

7 Q Why did you only publish one article?

8 A Because when I tried to reproduce -- this  
9 was a very, very small group. When I tried to reproduce it  
10 in a group that our statisticians told us would be a  
11 reasonable study, we couldn't get any response to  
12 Clonidine which is greater than chance, nor could we find  
13 a reproducible symptom cluster.

14 Q Are you familiar with any of the research  
15 done on the nicotine patch?

16 A I've read research.

17 Q Were they able to achieve statistical  
18 significance in any of that research?

19 A To which paper are you referring?

20 Q Any of the research you're familiar  
21 with on the nicotine patch.

22 A Some do; some don't.

23 Q So some of the research showed a  
24 statistically significant difference between the people  
25 who used the patch versus people who did not use the patch

1 as far as their ability to stop smoking cigarettes; is that  
2 correct?

3 A In some of the studies.

4 Q And was that also true for nicotine gum?

5 MR. KEMNA: Objection.

6 A It was true to a lesser degree.

7 Q And now there's an antidepressant that's  
8 now prescribed sometime for smoking cessation. What is the  
9 name of that antidepressant?

10 A It's a reformation of Wellbutrin.

11 Q What's it called?

12 A Zyprixin or something like that, but it  
13 is a reformation of Wellbutrin.

14 Q Which is an antidepressant?

15 A That is correct.

16 Q And there have been research studies on  
17 that which show statistical significance also; correct?

18 A There has been.

19 Q And what do those studies that are  
20 statistically significant conclude concerning the use of  
21 the antidepressant Wellbutrin?

22 A Smokers who use Wellbutrin at a certain  
23 dosage range which varies from study to study are able to  
24 discontinue smoking at a rate that the authors conclude is  
25 significantly higher than those who do not employ

1 Wellbutrin.

2 Q But even with the use of the Wellbutrin,  
3 I think it's called Zyban now, isn't it?

4 A That is correct.

5 Q But even with the use of the Zyban, and  
6 even though it was a statistically significant increase,  
7 the numbers were well below 50 percent of those who took  
8 the Zyban and then ultimately did quit smoking; correct?

9 A Your question's unclear to me. Could you  
10 restate it, please. Clarify.

11 Q Well, the people who took the Zyban, less  
12 than 50 percent of them were able to actually stop smoking;  
13 is that correct?

14 A I believe two studies reported that, or  
15 two studies that I saw.

16 Q That it was less than 50 percent of the  
17 people who took the Zyban were able to stop smoking;  
18 correct?

19 A In two of the studies.

20 Q But even that number that was actually  
21 significantly less than 50 percent success rate, that  
22 number was higher than the number of people in the control  
23 group who did not use Zyban; isn't that correct?

24 A That is correct.

25 Q Now, since you've taken the Broin

1 deposition in -- let's go back and look at the date. You  
2 do remember being present for the Broin deposition;  
3 correct?

4 A Yes, sir.

5 Q Since that deposition was taken on May  
6 30, 1997 -- and I'll just tell you that according to my  
7 copy, that's when it was taken -- have you been deposed in  
8 any other cases other than this one today, any other  
9 tobacco-related cases?

10 A What was that date again?

11 Q May 30, 1997.

12 A Yes.

13 Q What other tobacco-related cases have you  
14 been deposed in since then?

15 A Arch.

16 Q And when was that deposition?

17 A I believe it was in the autumn of last  
18 year.

19 Q And how long did that take?

20 A Less than a day.

21 Q Do you remember who took it, asked you  
22 the questions?

23 A I believe his name was Becknell or  
24 Bucknell.

25 Q Now, during the Broin deposition, I

1 believe you said that you charged approximately \$2,000 per  
2 day for a deposition; is that correct?

3 A That's approximately correct.

4 Q Is that still what you charge?

5 A I believe it's 2,200 for an in-town  
6 deposition.

7 Q That's regardless of how long the  
8 deposition takes?

9 A That is correct.

10 Q For example, today we're starting at  
11 1:30; we probably won't be more than three hours or so, but  
12 your fee will still be \$2,000 for that; is that correct?

13 A That is correct.

14 Q I mean \$2,200; correct?

15 A That is correct.

16 Q And this case you're being deposed on  
17 today is the Engle case; are you aware of that?

18 A Yes.

19 Q Do you know what the Engle case is about?

20 A My knowledge of the Engle case is that  
21 one of the issues is the alleged addictiveness of nicotine  
22 or other constituents of tobacco.

23 Q Other than knowing that that's one of the  
24 allegations, do you know anything else about the Engle  
25 case?

1 A I do not.

2 Q Have you read any depositions or reviewed  
3 any documents from the Engle case?

4 A I have not.

5 Q Have you prepared any reports for the  
6 Engle case?

7 A Yes.

8 Q And I'm talking about other than your  
9 disclosure statement where you explained what you're going  
10 to testify about.

11 A No.

12 Q Other than the disclosure statement, you  
13 haven't prepared any reports for the Engle case; is that  
14 correct?

15 A That is correct.

16 Q Approximately how many hours have you  
17 spent working on the Engle case?

18 A I'm reviewing my billing statements. In  
19 reviewing the billing statements, it's about 70 hours.

20 Q What is your hourly fee other than for  
21 depositions?

22 A Review of material, \$185 an hour.

23 Q And what material have you reviewed for  
24 the Engle case?

25 A The literature, documents in my

1 possession, mostly the literature.

2 Q And you reviewed the 1988 Surgeon  
3 General's report?

4 A Yes, sir.

5 Q And that was the Surgeon General's report  
6 that was related to smoking and addiction; is that correct?

7 A It was related to smoking, an opinion  
8 that it was addicting.

9 Q The Surgeon General referred to cigarette  
10 smoking as addictive; correct?

11 A That is not correct.

12 Q Was there a conclusion in the Surgeon  
13 General's report that cigarette smoking is addictive?

14 A Yes.

15 Q And you disagree with that conclusion;  
16 correct?

17 A Yes.

18 Q Do you treat patients as part of your  
19 practice as a physician as a psychiatrist?

20 A Yes.

21 Q Approximately how many patients do you  
22 treat in a given year?

23 A I'm in the office three to five days a  
24 week, and I see twelve to fifteen patients a day; plus I  
25 supervise directly or indirectly maybe another 30 to 40

1 patients a day.

2 Q How many patients do you see on a weekly  
3 basis, the same one?

4 A You're asking me how many times I need to  
5 see the patient more than once?

6 Q Some of them you do need to see more than  
7 once; correct?

8 A That's rare.

9 Q Okay. So then the count then isn't  
10 necessarily a duplicated count then. So if we just took  
11 twelve to fifteen people three to five times a day, you see  
12 somewhere between 60 and 75 patients a week and you times  
13 that by 52 and that comes up with a reasonable estimate of  
14 how many patients you see in a year?

15 A That would be a reasonable estimate.

16 Q How long have you been seeing  
17 approximately that many patients per year, how many years?

18 A Eight years.

19 Q And during that period of time -- well,  
20 not during that period of time; but approximately what  
21 percentage of your patients are cigarette smokers?

22 A I don't know.

23 Q Some of your patients are cigarette  
24 smokers?

25 A Yes.



1 Q How are you able to find that out if you  
2 do find it out?

3 A We have a standardized form for the first  
4 visit, and that is one of the questions. It's a form I  
5 wrote when I was a resident.

6 Q Do you ever advise any of them to stop  
7 smoking?

8 A No.

9 Q Do any of your patients ever say that  
10 they would like assistance in quitting smoking?

11 A Some.

12 Q Approximately how many in a given year  
13 will tell you they would like some assistance in quitting  
14 smoking?

15 A It would be rare.

16 Q By rare, what do you mean; one every  
17 week, one every year; what do you mean?

18 A Less than one a month.

19 Q And about how many of the people who you  
20 see who smoke just continue to smoke cigarettes?

21 A I didn't understand your question.

22 Q How many of the people that you have as  
23 patients who smoke continue to smoke cigarettes and just  
24 don't quit?

25 A I don't know.

1 Q Do you know whether it's 90 percent of  
2 the people that smoke that you see, they just continue to  
3 smoke without quitting?

4 A I don't know.

5 Q You don't have any idea; is that correct?

6 A That is correct.

7 Q That's not something you keep track of;  
8 right?

9 A That is correct.

10 Q Have you ever prescribed a nicotine patch  
11 to any of your patients?

12 A Yes.

13 Q You said yes?

14 A Yes.

15 Q Have you ever prescribed nicotine gum to  
16 any of your patients?

17 A Yes.

18 Q Have you ever prescribed Zyban to any of  
19 your patients?

20 A No.

21 Q You said no to Zyban?

22 A Yes.

23 Q Why have you never prescribed Zyban?

24 A Generally the only reason I prescribe the  
25 patch or the gum is if I'm covering the practice of another

1 doctor, and no doctor that I'm covering has yet prescribed  
2 Zyban.

3 Q And you have never prescribed the patch  
4 to a single one of your own patients?

5 A I've never initiated it.

6 Q When you say never initiated it, what do  
7 you mean? What's the distinction between that and  
8 prescribing it?

9 A As I said earlier, I will continue it if  
10 I have the responsibility of another doctor's patient whom  
11 I'm covering for a short period of time.

12 Q Do you ever recommend the nicotine patch  
13 to any patient?

14 A No.

15 Q Do you ever recommend against the  
16 nicotine patch to any patient?

17 A No.

18 Q Do you ever recommend Zyban to any  
19 patient?

20 A No.

21 Q Do you ever recommend against Zyban?

22 A No.

23 Q Have you ever had any patients who said  
24 they wanted to quit smoking but did not?

25 A Yes.

1 Q Do any of them tell you what happened  
2 when they tried to quit?

3 A Some.

4 Q And what do they tell you?

5 A Biggest complaint is they gained weight.

6 Q That's pretty much all they tell you, "I  
7 gained weight," and that's it?

8 A No, this is the most common complaint.

9 Q You mean of those who quit for a while,  
10 they complained to you that they gained weight?

11 A That's the most common complaint.

12 Q Do any people who don't have a problem  
13 with weight gain ever try to quit smoking in your  
14 experience?

15 A Yes.

16 Q Are they all successful?

17 A Some.

18 Q Do you have any who are not successful?

19 A Yes.

20 Q What percentage of them are not  
21 successful?

22 A I don't have those numbers.

23 Q That's just not something you track;  
24 correct?

25 A That is correct.

1 Q Have you ever had any patients who had  
2 lung cancer?

3 A Yes.

4 Q Have you ever had any patients who had  
5 lung cancer who smoked cigarettes?

6 A Yes.

7 Q Have you ever had any patients who had  
8 lung cancer who continued to smoke cigarettes even after  
9 they were diagnosed with the lung cancer?

10 A Yes.

11 Q And even after they were told to quit  
12 smoking?

13 A Yes.

14 Q Have you ever had any patients who had a  
15 lung removed and continued to smoke cigarettes?

16 A Yes.

17 Q Have you ever had any patients with  
18 Berger's disease?

19 A Yes.

20 Q What is Berger's disease?

21 A Berger's is a disease of the peripheral  
22 vasculature.

23 Q And does cigarette smoking cause Berger's  
24 disease?

25 MR. KEMNA: Objection. Dr. Giannini

1 is not offered as an expert on the causation of disease  
2 generally or specifically with respect to Berger's disease.

3 Q If you don't know, you can say, Doctor.  
4 Does cigarette smoking cause Berger's disease?

5 A To the best of my knowledge, cigarette  
6 smoking is a risk factor.

7 Q Is there a difference between risk factor  
8 and cause?

9 A Yes.

10 Q So are you saying that you don't know  
11 whether cigarette smoking causes Berger's disease?

12 A I'm saying no one knows.

13 Q Does anyone know whether cigarette  
14 smoking causes lung cancer, in your opinion?

15 MR. KEMNA: Objection. Dr. Giannini  
16 is not being offered on the causation issue of any disease.  
17 It's inappropriate to continue to ask him questions when  
18 you know he's not going to be an expert in the field.

19 Q Let me back up. What's the basis of your  
20 opinion that no one knows whether cigarette smoking causes  
21 Berger's disease?

22 MR. KEMNA: Objection. Dr. Giannini  
23 is not being offered as an expert on the causation of  
24 Berger's disease. That's very clear. This is an  
25 unproductive line of questioning.

1 MR. HOAG: I appreciate you telling  
2 him you don't want him to answer the question, but he's  
3 already said that no one knows.

4 Q So what I'm asking you, Doctor, is -- and  
5 I'll preserve your objection so we don't have to waste time  
6 with it constantly in there. What I'm asking you is what  
7 is the basis of your opinion of no one knows whether  
8 cigarette smoking causes Berger's disease?

9 MR. KEMNA: Same objection regarding  
10 expertise.

11 A It's listed as a risk factor.

12 Q The basis of your opinion that no one  
13 knows whether cigarette smoking causes Berger's disease is  
14 that it's listed as a risk factor?

15 MR. KEMNA: Same objection regarding  
16 expertise.

17 Q Is that what you're saying?

18 A When I last reviewed vascular disease,  
19 the total group of vascular diseases, smoking was listed as  
20 a risk factor in some and not others.

21 Q And what about lung cancer, what was  
22 cigarette smoking listed as as it relates to lung cancer?

23 MR. KEMNA: Same objection. Dr.  
24 Giannini is not being offered as an expert on lung cancer.

25 MR. HOAG: You can answer.

1 A Some authors list it as a risk factor.  
2 Some list it as a causation.

3 Q And you don't know one way or the other  
4 whether it's the cause of lung cancer or not; correct?

5 A I lack expertise in epidemiology,  
6 oncology and pulmonology to decide between which of the  
7 articles, if either or both or neither are correct.

8 Q So you don't believe there's a consensus  
9 of medical opinion that cigarette smoking causes lung  
10 cancer; is that correct?

11 MR. KEMNA: Objection.

12 A I'm testifying since I'm neither an  
13 expert in epidemiology, oncology or pulmonology, I cannot  
14 discriminate between the different articles and differing  
15 conclusions.

16 Q Is there a controversy as to whether or  
17 not cigarette smoking causes lung cancer, Doctor?

18 MR. KEMNA: Objection. Dr. Giannini  
19 is not being offered as an expert on lung cancer or the  
20 controversy that you have within your question. This is  
21 totally unproductive, John.

22 Q You can answer, Doctor.

23 A I have not read sufficiently in this area  
24 to know if there's a controversy one way or the other.

25 Q How many of the patients that you see



1 have read sufficiently in the area to know whether there's  
2 a controversy as to whether cigarette smoking causes lung  
3 cancer?

4 A You're asking me which of my patients  
5 have enough knowledge of physiology and medicine to make an  
6 informed opinion?

7 Q Yes.

8 A Then we're limiting my patients to  
9 physiologists, physicians and Master's level nurses.

10 Q So you have to have those kind of  
11 credentials to actually be able to know whether or not  
12 cigarette smoking causes lung cancer?

13 MR. KEMNA: Objection. Dr. Giannini  
14 is not offered as an expert on the awareness of the public  
15 to the risks of cigarette smoking.

16 Q You can answer.

17 A Could you please repeat.

18 MR. HOAG: Could you read back the  
19 question. And we'll preserve your objection.

20 (Whereupon the record was read as requested.)

21 A I wouldn't know. I'm not an expert in  
22 educational theory.

23 Q You have had patients who have had  
24 Berger's disease; correct?

25 A I have.

1 Q Are any of those patients smokers?

2 A I can speculate that they weren't, but I  
3 cannot say for certain.

4 Q Now, those smokers that have lost a lung  
5 and continue to smoke cigarettes, does that indicate in any  
6 way to you that cigarette smoking is addictive?

7 A No.

8 Q Is there anything about cigarette smoking  
9 and people's behavior or response to smoking that indicates  
10 to you in any way, even if it's the slightest, tiniest way,  
11 that cigarette smoking may be addictive?

12 A No.

13 Q You would agree that there is research  
14 showing that animals will self-administer nicotine;  
15 correct?

16 A I will agree that there have been studies  
17 showing animals can be taught to self-administer nicotine.

18 Q And you consider nicotine to be a drug;  
19 correct?

20 A Yes.

21 Q And nicotine crosses the blood barrier,  
22 gets to the brain within ten seconds; correct?

23 MR. KEMNA: Objection.

24 A Which blood barrier do you mean?

25 Q Blood brain barrier.

1 A Yes, it does.

2 Q And do you agree that when cigarette  
3 smoke is inhaled, the nicotine first stimulates sensory  
4 nerve endings imbedded in the vicinity of the millions of  
5 air sacs of the lung?

6 A Repeat that again, please.

7 Q Do you agree that when cigarette smoke is  
8 inhaled, the nicotine first stimulates sensory nerve  
9 endings imbedded in the vicinity of the millions of air  
10 sacs of the lungs?

11 A When you say first, you mean first in  
12 relation to what do you mean second?

13 Q Well, first means first; it comes before  
14 other things.

15 A I need to know in relation to what. Your  
16 question makes no -- no offense, but your question makes no  
17 sense to me. First --

18 Q As far as stimulation of nerve endings,  
19 before it goes anywhere else.

20 A That it goes to the lungs first before it  
21 goes anywhere else?

22 Q Right. It stimulates sensory nerve  
23 endings imbedded in the vicinity of the millions of air  
24 sacs of the lungs. Do you agree?

25 A No. No. Of course not.

1 Q And why do you disagree with that?

2 A Unless you have a hole in your chest,  
3 there's no way it's going to the lungs first.

4 Q Well, what nerve endings does it  
5 stimulate before it stimulates the nerve endings in the  
6 vicinity of the millions of air sacs of the lungs?

7 A The gum, the palate, the tongue, the  
8 pharynx, in some people the sinuses.

9 Q Okay. So are you saying then the  
10 nicotine stimulates all of those nerve endings and then it  
11 finally gets to the lungs; is that right?

12 A I'm saying that it has to go through the  
13 mouth and the throat and the bronchi before it can hit the  
14 lungs. That's basic human physiology.

15 Q Are you saying that it stimulates all the  
16 nerve endings in all those parts of the body prior to the  
17 time it gets to the lungs; is that correct?

18 A I'm saying yes.

19 Q Do you agree that within seconds the  
20 stimulation produces a powerful reflux effect consisting of  
21 a brief, abrupt fall in heart rate and blood pressure?

22 MR. KEMNA: Objection to form.

23 A Okay. There's one word. Ask your  
24 question, please, but ask very slowly because there's one  
25 word that I have to ask you to make sure I heard you

1 correctly. Could you repeat the question slowly?

2 Q Okay. Do you agree that within seconds --

3 A Slowly, slowly.

4 Q -- the stimulation of nerve endings  
5 produces a powerful reflux -- I'm sorry, reflex.

6 A That's the word.

7 Q Effect consisting of a brief, abrupt fall  
8 in heart rate and blood pressure?

9 A Okay. Now that you corrected it, please  
10 give me the question one more time.

11 Q Do you agree that within seconds the  
12 stimulation of nerve endings produces a powerful reflex.  
13 effect consisting of a brief, abrupt fall in heart rate and  
14 blood pressure?

15 MR. KEMNA: Objection to form.

16 A I don't know if it's brief or abrupt, but  
17 there is a change in blood pressure, and it's downward.

18 Q Do you agree that the nicotine stimulates  
19 a generalized relaxation of the body musculature?

20 MR. KEMNA: Objection to form.

21 A Yes.

22 Q Do you agree that at the same time it  
23 creates an arousal of the brain?

24 MR. KEMNA: Objection to form.

25 A By arousal you mean? That's too general

1 for me to answer.

2 Q What does the word arousal mean to you as  
3 it applies to the brain?

4 A Well, see, arousal means many things.  
5 It's not that I don't want to answer. I just don't  
6 understand your question. If you could be a little more  
7 specific.

8 Q Well, does it create any kind of arousal  
9 based on your definition of arousal of the brain?

10 A Do you mean general arousal? Do you mean  
11 sexual arousal? What do you mean by arousal?

12 Q I mean any kind of arousal of the brain.

13 A Yes, it produces some kind of arousal.

14 Q What kind of arousal of the brain does  
15 nicotine produce?

16 A Generalized.

17 Q Pardon me?

18 A Generalized.

19 Q A generalized arousal?

20 A Yes.

21 Q What does it generally arouse? What does  
22 it cause to happen in the brain?

23 A There's change in electrical activity,  
24 and there is a change in neurochemical release and tone.

25 Q It increases the dopamine level; correct?

1 A Yes. Well, it doesn't increase the level  
2 per se.

3 Q What does it do to the dopamine; what  
4 effect does it have on dopamine?

5 A It increases potential for dopamine  
6 release.

7 Q In the brain; correct?

8 A In the brain, yes. All my questions are  
9 limited to the brain.

10 Q And there is a substance in cigarette  
11 smoke that is an MAO inhibitor; correct?

12 A It may be an MAO inhibitor.

13 Q Which means the dopamine that's released  
14 is going to stay in the brain longer; correct?

15 A That is correct.

16 Q It also increases the serotonin level in  
17 the brain; correct?

18 A That is also correct.

19 Q It increases the epinephrine level;  
20 correct?

21 A That is correct.

22 Q And what is dopamine?

23 A Dopamine is a catecholamine utilized as a  
24 transmitter, neurotransmitter, in the body and brain.

25 Q And it creates feelings of pleasure,

1 doesn't it?

2 A It can.

3 Q Cocaine releases dopamine, doesn't it?

4 A I'm sorry?

5 Q Cocaine releases dopamine?

6 A That is correct.

7 Q And serotonin, what does it do?

8 A Serotonin is an indolamine that has  
9 multiple functions throughout the body.

10 Q And what kind of feelings does serotonin  
11 create?

12 A It has different feelings.

13 Q What would those be?

14 A It could cause diarrhea. It could cause  
15 an increased heart rate. It could cause flushing. It can  
16 cause anxiety, and it can cause a feeling of well-being.

17 Q The feeling of well-being, that's the  
18 reason it's an antidepressant, or that's the reason  
19 antidepressants like Prozac and Zoloft work; correct?

20 MR. KEMNA: Objection to form.

21 A I don't understand your question.

22 Q Using Prozac, for example, the serotonin  
23 reuptake inhibitor?

24 A Yes.

25 Q Which means that it causes the brain to



1 have a better supply of serotonin; correct?

2 A I'm not sure if it's better.

3 Q More of a supply of serotonin?

4 A Yes.

5 Q Which is the same thing that cigarette  
6 smoking does; correct?

7 A It's never been proven that cigarette  
8 smoking is a serotonin reuptake inhibitor.

9 Q I'm not talking about serotonin reuptake  
10 inhibitor now, and I appreciate your clarification. What  
11 I'm talking about is cigarette smoke. The nicotine causes  
12 an increase in the amount of serotonin that's available;  
13 correct?

14 A That is correct. Excuse me; I have to  
15 take a break.

16 MR. KEMNA: Let's take five, John.

17 (Whereupon a brief recess was taken.)

18 Q (BY MR. HOAG) Did you ever prescribe a  
19 medication called Effexor?

20 A Yes.

21 Q That's an antidepressant; is that  
22 correct?

23 A Yes.

24 Q That's prescribed frequently for people  
25 with severe depression?

1 A I'm not sure what you mean by severe.

2 Q Well, you don't operationalize the term  
3 severe depression versus moderate or mild?

4 A Yes.

5 Q I guess I mean more than moderate  
6 depression. Effexor is frequently prescribed for people  
7 who have more than a moderate depression; is that correct?

8 A It can be.

9 Q Now, Effexor contains Epinephrine, and  
10 it's also a serotonin reuptake inhibitor, isn't it?

11 MR. KEMNA: Objection.

12 A It does not contain norepinephrine.

13 Q What does it contain?

14 A Effexor acts as noradrenergic and  
15 serotonergic reuptake inhibitor.

16 Q Would you repeat that, please?

17 A Noradrenergic and serotonergic reuptake  
18 inhibitor.

19 Q Okay. The second part was serotonin;  
20 right?

21 A No, serotonergic.

22 Q Serotonergic reuptake inhibitor?

23 A Yes, sir.

24 Q What is that?

25 A Serotonergic reuptake means it blocks the

1 reuptake of serotonin so that serotonin can be metabolized.

2 Q So it makes serotonin more available to  
3 the brain; correct?

4 A That is correct.

5 Q Prozac does that too; correct?

6 A That is correct.

7 Q Zoloft does that, too; correct?

8 A That is correct.

9 Q All of those are antidepressants;  
10 correct?

11 A That is also correct.

12 Q Prescribed to millions of people in the  
13 United States each year; correct?

14 A As far as I know, yes.

15 Q And nicotine increases the availability  
16 of serotonin to the brain, too; correct?

17 MR. KEMNA: Objection.

18 A That is correct.

19 Q Now, you have -- have you ever treated  
20 patients who are using cocaine; do you ever treat them and  
21 assist them in stopping the use of cocaine?

22 A Yes.

23 Q And are any of those people cigarette  
24 smokers?

25 A Some.

1 Q What percentage of the cocaine users are  
2 cigarette smokers, from your experience?

3 A I've never done a study.

4 Q Isn't it a very small percentage who  
5 don't smoke cigarettes?

6 A As I said, I've never done a study.

7 Q Do any of the cocaine users who stop  
8 using cocaine continue to smoke cigarettes?

9 A Some do; some don't.

10 Q What percentage of the cocaine users whom  
11 you have treated who actually stopped using cocaine  
12 continue to smoke cigarettes?

13 A I do not have the exact numbers.

14 Q Do you have any general numbers?

15 A No.

16 Q Do you ever provide treatment to people  
17 who are considered to be alcoholics?

18 A Yes.

19 Q You said yes?

20 A Yes.

21 Q And what percentage of those individuals  
22 smoke cigarettes?

23 A I do not have those numbers.

24 Q Isn't it true that the vast majority of  
25 people who are alcoholics are also cigarette smokers?

1 MR. KEMNA: Objection.

2 A That is not true.

3 Q And what do you base that on?

4 A Vast is not a statistical term.

5 Q Oh, vast is not a statistical term;  
6 that's what you base it on?

7 A You're using incorrect usage.

8 Q Well, I guess I would wonder then how do  
9 you know whether or not I'm right or not with the word vast  
10 if you don't know what the word vast means?

11 A Vast means large, all encompassing.

12 Q You mean you define vast to mean 100  
13 percent?

14 A No, it means vast, large, all  
15 encompassing,. All-encompassing means everything.

16 Q Okay. So to you the term vast majority  
17 means 100 percent; is that right?

18 MR. KEMNA: Objection.

19 A You're misusing the word vast. Vast is  
20 an adjective and cannot modify majority.

21 Q Okay. So vast has no meaning at all; is  
22 that right?

23 MR. KEMNA: Objection.

24 A I do not edit dictionaries, but vast  
25 correctly should be limited to geographical or astronomical

1 spaces. It's not meant to modify quantitative measurements  
2 except for astronomical or geographical distances.

3 Q Okay. Let's try to be more specific  
4 then. You'll agree that more than 50 percent of the people  
5 who are alcoholics are also cigarette smokers; correct?

6 A Yes.

7 Q You'll agree that more than 75 percent of  
8 the people who are alcoholics are also cigarette smokers;  
9 correct?

10 A In some countries.

11 Q How about the United States?

12 A Yes.

13 Q You say yes?

14 A Yes.

15 Q And did you ever successfully treat a  
16 person who is alcoholic but the person who was successfully  
17 treated for alcoholism continued to smoke cigarettes?

18 A Some do; some don't.

19 Q What percentage do continue to smoke  
20 cigarettes after they've stopped drinking alcohol?

21 MR. KEMNA: Objection.

22 A I do not have those figures.

23 Q Do you agree that the first changes in  
24 electroencephalogram, or EEG, readings which measure the  
25 electrical activity in the brain, of course, occur before

1 nicotine reaches the brain; and I'm specifically referring  
2 to the effect that nicotine has? Do you agree with that?

3 MR. KEMNA: Objection to form.

4 A First of all, your question is based on a  
5 supposition that the EEG measures electrical activity of  
6 the brain. It does not. It measures only the first  
7 centimeter or two.

8 Q It measures only the first centimeter or  
9 two of the brain?

10 A Yes.

11 Q Using your definition of EEG?

12 A It's not my definition of EEG. It's the  
13 manufacturer's. That's all EEGs are capable of doing.

14 Q Well, your understanding of EEG.

15 A I cannot answer. It's not my  
16 understanding. That's what EEGs do.

17 Q Using your absolute certainty of your  
18 understanding of EEG --

19 A I cannot respond to it. If you will ask  
20 me the question of what EEGs actually do, I will respond.

21 Q Do you agree that -- so you're an expert  
22 on EEGs; is that correct?

23 A No.

24 Q But you have absolutely certainty about  
25 what an EEG does; correct?

1 MR. KEMNA: Objection.

2 A It's one of the tools we learn in medical  
3 school.

4 Q But you never learned in medical school  
5 whether cigarette smoking causes any disease; is that  
6 correct?

7 MR. KEMNA: Objection. Asked and  
8 answered on a number of occasions.

9 Q Is that correct?

10 MR. KEMNA: Same objection.

11 A Please repeat the question.

12 Q But you never learned in medical school  
13 that cigarette smoking causes disease; is that correct?

14 MR. KEMNA: Objection. Asked and  
15 answered.

16 A I'll refer you to my previous answer.

17 Q But you did learn in medical school with  
18 absolutely certainty exactly how an electroencephalogram  
19 works and exactly how many centimeters of the brain that it  
20 effects; is that correct?

21 A If you will recall, I took neurosurgery  
22 in medical school. The EEG is an important component of  
23 neurosurgery.

24 Q You did take courses on causation of  
25 disease in medical school, too, didn't you?



1 MR. KEMNA: Objection. Asked and  
2 answered.

3 Q I do recall you saying that; am I right?

4 MR. KEMNA: Objection. Asked and  
5 answered.

6 A I have previously responded to your  
7 question.

8 Q Yes, and you said you took courses in  
9 causation, just to clarify the situation. As far as logic  
10 and reason are concerned.

11 Now, let me try to ask the question again, and I'm  
12 sure that you'll comment if there's anything in the wording  
13 that troubles you in any way.

14 • Would you agree that the first changes in an  
15 electroencephalogram, EEG readings, as it relates to  
16 nicotine, occur before nicotine reaches the brain?

17 MR. ARCHIE: Objection to form.

18 A It has been reported.

19 Q Is that your answer?

20 A It has been reported.

21 Q By whom?

22 A I do not know.

23 Q So you don't know whether or not that's  
24 true or not; is that correct?

25 A All I can say is it has been reported. I

1 cannot give you the author.

2 Q Will you -- do you accept that report as  
3 being reliable and accurate?

4 A I can neither accept nor not accept. In  
5 science it's not -- things are not taken on faith. There's  
6 a series of reports, and over time the reports generate  
7 certain hypotheses which are then tested.

8 Q So these reports that you're aware of, do  
9 you know whether or not they've been tested over time?

10 A I do not.

11 Q Do you agree that both muscle relaxation  
12 and EEG arousal are reinforced by nicotine action on  
13 neuronal circuits in the brain and spinal cord?

14 MR. KEMNA: Objection to form.

15 A When you say reinforced, you mean?  
16 Reinforced has multiple definitions in medicine. I need to  
17 know in which context you're using it.

18 Q What are your numerous definitions of  
19 reinforced?

20 A Again, these are not my numerous  
21 definitions. These are accepted definitions.

22 Q What are these accepted definitions  
23 of reinforced that you're familiar with, Doctor?

24 A Reinforced is setting up a behavioral  
25 pattern. Reinforced can mean a neuronal pattern.

1 Reinforced can mean an additive or multiplicative or  
2 synergistic effect.

3 Q Okay. In this instance I'm referring to  
4 the additive effect. Can you answer the question?

5 MR. KEMNA: Objection to form.

6 A I need you to repeat the question.

7 MR. HOAG: Can the court reporter  
8 repeat the question I asked?

9 (Whereupon the record was read as requested.)

10 Q I'll just start over. It's confusing to  
11 go backward that many times. Do you agree, Doctor that,  
12 both muscle relaxation and EEG arousal are reinforced,  
13 meaning there's an additive effect, by nicotine action on  
14 neuronal circuits in the brain and spinal cord?

15 MR. KEMNA: Objection to form.

16 A Okay. Now I'm starting to understand  
17 your question. Okay. The additive effect, to what is it  
18 being added; nicotine is being added to what?

19 Q The nicotine is being added to its  
20 initial effect. Its initial effect being muscle relaxation  
21 and EEG arousal. That effect is then reinforced by further  
22 nicotine action on neuronal circuits in the brain and  
23 spinal cord; do you agree?

24 MR. KEMNA: Objection to form.

25 A Yes.

1 Q Do you agree that nicotine causes the  
2 brain to release more adrenalin?

3 A Yes.

4 Q And dopamine; correct?

5 MR. KEMNA: Objection.

6 A Yes.

7 Q And that those two substances act as  
8 stimulants?

9 A Yes.

10 Q And dopamine, of course, also enhances  
11 feelings of pleasure and well-being; correct?

12 MR. KEMNA: Objection.

13 A It can.

14 Q Have you reviewed any tobacco industry  
15 internal documents?

16 A Yes.

17 Q Have you reviewed internal documents  
18 related to research concerning the effects of nicotine?

19 A Yes.

20 Q Have you reviewed any of the research of  
21 Dr. Frank Gullotta?

22 A Please spell that name.

23 Q G-U-L-L-O-T-T-A, I believe.

24 A I may have, but I have no recollection of  
25 seeing that name.

1 Q Have you reviewed research done at  
2 Battelle called the fate of nicotine in the body?

3 A Yes.

4 Q And is there anything in that research  
5 that indicates to you that nicotine may be addictive?

6 A No.

7 Q Is there anything in any of the tobacco  
8 industry internal documents that indicates to you that  
9 nicotine may be addictive?

10 MR. KEMNA: Objection.

11 A Not those that I have reviewed.

12 Q And how did you come to review internal  
13 documents? Were they shown to you during depositions?

14 A They were made available to me June 17 of  
15 this year.

16 Q By whom?

17 A Joseph Hunt.

18 Q And who is Joseph Hunt?

19 A He's an attorney.

20 Q Who did Joseph Hunt represent?

21 MR. HUNT: I'm on the line. Joseph  
22 Hunt; I represent the Brown & Williamson Tobacco  
23 Corporation.

24 MR. HOAG: Well, I really wasn't  
25 asking you.

1 MR. HUNT: I just wanted to clarify  
2 the record, and he may not know who all I represent; and  
3 you're asking about me, so I'm going to say who I  
4 represent.

5 MR. HOAG: Well, I really --

6 MR. HUNT: Even though I stated it at  
7 the beginning.

8 MR. HOAG: See, I'm not asking you;  
9 I'm asking the witness on the record here during the  
10 deposition. I'm not taking your deposition.

11 MR. HUNT: You want to object. Go  
12 ahead and ask him.

13 MR. HOAG: Well, I did ask him and  
14 you interrupted. All I wanted was an answer from the  
15 witness.

16 Q And who does Joseph -- I forgot your last  
17 name. Who does he represent?

18 A I assume you're speaking of Mr. Hunt?

19 Q Yeah, who does Mr. Hunt represent?

20 A To the best of my knowledge, Brown &  
21 Williamson.

22 Q And why did he send you those documents  
23 on June 17, if you know?

24 A To review.

25 Q Was it to review in preparation for the

1 Engle case?

2 A Yes.

3 Q Do you have those documents with you?

4 A Yes.

5 Q And can you identify those documents,  
6 please?

7 A I'm not sure what you mean by identify.

8 Q Well, how many documents do you have with  
9 you?

10 A Fourteen.

11 Q And can you identify those fourteen  
12 documents, please?

13 A Yes, Final Report on Project Hippo I;  
14 Report No. 1 regarding Project Hippo; Final Report on  
15 Project Hippo II. And I have one I cannot read the title.  
16 The photocopying is not good. I can read the report but  
17 not the title.

18 Q What's the report say?

19 A It says, first word I understand and it  
20 says then, so blank of report in Project Hippo, blank,  
21 blank and Project Hippo 2, blank, blank.

22 Q Okay. Next.

23 A Then there's a, all I can read is  
24 "telephone conversation" and basically that whole report  
25 was unreadable, so I couldn't consult it.

1 Q Okay. What was next?

2 A Fate of Nicotine in the Body.

3 Q Who's the author of that?

4 A Centre de recherche, de Gendre.

5 Q Okay. Who's next?

6 A Outgoing cable to Mr. McCormick from Ms.

7 Yeaman. Incoming cable to this individual Yeaman from Mr.

8 McCormick. Then a letter to Mr. Yeaman from Terry or

9 Tommy. Then a report, and I cannot read the title. Then a

10 memo to the chairman from Sir Charles Ellis. Then a note

11 from Mr. Cutchins.

12 Then Smoking and Health, Surgeon General Publication

13 1103. It includes the Advisory Committee, the committee

14 staff and Chapter 2.

15 Next one, Memo A.286 Visit to Battelle Institute,

16 Geneva, 8th and 9th August, 1963. A letter to Sir Charles

17 from Battelle Institute, signed, looks like Welk.

18 Q Is that all of them?

19 A No. Then Review of Activities of the

20 Tobacco Research Council 1963 to 1966.

21 And that concludes the documents which I received.

22 Q Okay. I'd like to just have those marked

23 as a composite exhibit, Plaintiff's Exhibit 1.

24 A Okay. And in addition I received, which

25 was not part of those documents, under separate cover, a



1 letter from Robert Hockett of the Tobacco Industry Research  
2 Committee to Peter Hamill, staff medical coordinator,  
3 Surgeon General's Advisory Committee on Smoking and Health,  
4 several pages, more than several pages. The first page Dr.  
5 Hockett informs Dr. Hamill that he's sending him a list of  
6 references for study on the possible beneficial effects of  
7 tobacco, and he informs Dr. Hockett that he may find this  
8 to be of some interest for further study; and then there  
9 follows a bibliography of 25 separate references assembled  
10 by the Tobacco Industry Research Council.

11 Then there follows another page of references,  
12 unnumbered, but it looks like it's approximately 20  
13 references. There follows another page of references,  
14 unnumbered, again approximately 15 references. There  
15 follows another page of references, approximately 20  
16 additional references in English, French, German, as well  
17 as a Scandinavian reference in English. There follows five  
18 more references from German and American sources.

19 That concludes my papers.

20 MR. HOAG: Okay. All those that  
21 you've listed, I'd like to have those put together as  
22 composite Plaintiff's Exhibit 1 and attached to his  
23 deposition.

24 MR. KEMNA: John, you should have a  
25 list of all those documents that we provided to your office

1 last week.

2 MR. HOAG: Okay. I just want to get  
3 them attached to the deposition.

4 A Okay.

5 (Whereupon Plaintiff's Exhibit No. 1 was marked.)

6 Q Doctor, do you rely in any way on any of  
7 those documents as a basis for any of your opinions in  
8 Engle?

9 A No.

10 Q And did you bring any other documents  
11 with you other than those you have already identified?

12 A Yes.

13 Q What other documents did you bring with  
14 you?

15 A Tobacco Experimental Clinical Studies by  
16 Larson, Haag & Silvette, Chapter 1.

17 Q Other than that, did you bring anything  
18 else with you other than that and what you've already  
19 identified?

20 A Yes.

21 Q What else?

22 A By accident. This is not an article  
23 which I actually used. It got included. Do you want that  
24 name?

25 Q Yes.

1 A "Addiction, Dependence and Habitual  
2 Substance Use," by David Warburton.

3 Q Okay. Other than those that you've  
4 already named, did you bring any other documents with you?

5 MR. KEMNA: John, we have copies of  
6 the Doctor's billing statements that were provided to your  
7 office regarding the Engle case.

8 MR. HOAG: Okay. You have the  
9 billing statements.

10 Q Anything else other than the billing  
11 statements and the other things you've already named did  
12 you bring with you today?

13 A No, sir.

14 MR. HOAG: Okay. I'd like to get  
15 the, I think you said it was an article by Larson & Haag,  
16 did you say, H-O-G-G?

17 A H-A-A-G, and I'll just spell them for the  
18 court reporter as well as you, Larson, Haag, H-A-A-G and  
19 S-I-L-V-E-R-T-T-E. It's a chapter in a book that they  
20 jointly authored.

21 MR. HOAG: I'd like to have that  
22 marked as Plaintiff's Exhibit 2; and also the third thing  
23 you listed that I think you said you don't rely on, I'd  
24 like that listed as Plaintiff's Exhibit 3, and the billing  
25 records as Plaintiff's Exhibit 4.

1 (Whereupon the reporter marked for identification  
2 Plaintiff's Exhibits 2, 3 and 4.)

3 Q As to Plaintiff's Exhibit 2, which is the  
4 chapter from the book by Larson and Haag, H-A-A-G; that's  
5 so close to my last name I like to pronounce it  
6 differently; I'll just say Haag. Do you rely in any way on  
7 that book; on that chapter?

8 A No.

9 Q There's nothing in that chapter that  
10 helps you form a basis for any of your opinions today?

11 A No.

12 Q Why did you bring it with you today?

13 A Because I reviewed it in my preparation  
14 for this deposition.

15 Q But it didn't assist you in any way in  
16 your preparation?

17 MR. KEMNA: Objection.

18 A I don't know if it assisted me. It did  
19 not make me or assist me in forming a specific opinion.

20 Q Did it assist you in any way in preparing  
21 for the deposition?

22 A I can't think of a way. I'm not saying  
23 it didn't. I just can't think of a way in which it did.

24 Q Okay. And Plaintiff's Exhibit 3, did  
25 that assist you in any way in preparing for the deposition?

1 A As I said earlier, I put this in a bag  
2 that I brought, a backpack, I brought all these materials.  
3 It must have been in the backpack already. It wasn't used  
4 at all.

5 Q Okay. You didn't even review that?

6 A No. That was included accidentally. It  
7 was probably in the backpack when I packed it.

8 MR. HOAG: Okay. I don't have any  
9 other questions. Do any of you have any questions?

10 MR. KEMNA: John, let's take a break  
11 for a moment. We'll come back in about five.

12 (Whereupon a brief recess was taken.)

13 DIRECT EXAMINATION:

14 BY MR. KEMNA

15 Q Dr. Giannini, just a few questions.

16 A Yes, sir.

17 Q The documents that have been marked  
18 collectively as Plaintiff's Exhibit 1 consist of documents  
19 that you were sent on, well, with the cover letter, June  
20 17, 1998?

21 A That's correct.

22 Q And you've had an opportunity to review  
23 these documents; is that correct?

24 A That is correct.

25 Q Is there anything in those documents that

1 in your opinion would change any views that you have  
2 regarding the question of whether cigarette smoking should  
3 be considered addictive?

4 A No, sir.

5 Q To the extent you had an opportunity to  
6 review those documents previously marked as --

7 MR. HUNT: I was afraid I might have  
8 been cut out. I got cut off one time before. That's one  
9 reason I wanted to identify myself to you on the record  
10 because when I first did come on the record earlier at the  
11 deposition, you weren't yet on the phone, Mr. Hoag. So I  
12 just want to make sure that I haven't been cut off again.

13 MR. KEMNA: John, Jodie, you're both  
14 on the line, and, in fact, I had started asking questions  
15 of Dr. Giannini not knowing that you could not hear me.

16 MR. HUNT: I heard some stuff. I  
17 heard you ask him about the documents and if they were sent  
18 to him and if he had a chance to review them, and I didn't  
19 hear anything after that.

20 MR. HOAG: I also didn't hear  
21 anything after that.

22 MR. KEMNA: Let me just go back on  
23 with questions and answers from that point then.

24 Q Doctor, you've already indicated that  
25 you've had an opportunity to review the documents marked as

1 Plaintiff's Exhibit 1; is that correct?

2 A That is correct.

3 Q And in your opinion, is there anything  
4 within that collection of documents that you have reviewed  
5 that would affect your opinion that cigarette smoking  
6 should not be considered an addiction?

7 MR. KEMNA: Can you repeat that  
8 question?

9 Q You've reviewed the documents  
10 collectively marked as Plaintiff's Exhibit 1. Is there  
11 anything contained in those documents that would change the  
12 opinion that you have expressed that cigarette smoking  
13 should not be considered an addiction?

14 A No, sir.

15 Q Is there anything about the documents  
16 collectively marked as Plaintiff's Exhibit 1 that you think  
17 would have been necessary as information to be disclosed to  
18 anyone at that time to resolve the question of whether  
19 cigarette smoking should be considered an addiction?

20 MR. HOAG: You cut out on several  
21 words for some reason. I only heard half the words you  
22 said in that last question. So if the court reporter  
23 could perhaps repeat it.

24 MR. KEMNA: Yes.

25 (Whereupon the record was read as requested.)

1 MR. HOAG: I'm going to object to the  
2 form of the question.

3 A There is no information in these  
4 documents that would have changed the knowledge in the  
5 scientific community at that time.

6 Q Dr. Giannini, if you are asked questions  
7 regarding the research disclosed within the documents  
8 marked as Exhibit 1 collectively at the trial of this  
9 matter, do you feel prepared to respond within your field  
10 of expertise regarding those documents?

11 A Yes, sir.

12 MR. HOAG: I'm going to object to the  
13 form of the question.

14 Q Doctor, you've seen your expert  
15 disclosure statement in this case?

16 A Yes, sir.

17 Q In your disclosure statement you agree,  
18 don't you, that it says that Dr. Giannini may also be asked  
19 to comment upon the opinions expressed by other witnesses  
20 to the extent that they relate to his areas of expertise?

21 A Yes, I see that.

22 Q Does that accurately reflect the  
23 expectation you have to testify regarding the issues in the  
24 trial of the Engle case?

25 A Yes, sir.



1 MR. KEMNA: That's all I have.

2 MR. HOAG: Okay.

3 CROSS EXAMINATION:

4 BY MR. HOAG

5 Q What opinions of other witnesses are you  
6 going to testify about?

7 A I'm not sure what opinions they're going  
8 to give.

9 Q Do you know who the witnesses are on the  
10 Engle case?

11 A No.

12 Q Have you read any depositions in the  
13 Engle case?

14 A No, sir.

15 MR. HOAG: Well, I will just object  
16 to any testimony this witness plans to provide on things  
17 that he doesn't know about today and move to strike any  
18 such testimony that's made in the future; and I'm done.

19 MR. HUNT: I have a question.

20 MR. HOAG: Well, wait a second. You  
21 just did that. You don't get a question after this.

22 MR. HUNT: Nobody asked me if I had a  
23 question and you just started in with your own question.

24 MR. HOAG: Well, I'm going to object,  
25 but go ahead.

1 CROSS EXAMINATION:

2 BY MR. HUNT:

3 Q Dr. Giannini, if you are asked questions  
4 at the trial of the Engle case concerning the documents  
5 that have been marked collectively as Plaintiff's Exhibit 1  
6 at this deposition, do you intend to give your opinion that  
7 you have about those documents based on your review of  
8 those documents?

9 A Yes, sir.

10 MR. HUNT: I have no further  
11 questions.

12 MR. HOAG: All right. I'm going to  
13 object to the Doctor providing any opinions concerning  
14 these documents because this was not disclosed on the  
15 disclosure statement anywhere that he was going to review  
16 internal documents and provide opinions concerning what  
17 those internal documents meant; and when I asked him if he  
18 relied in any way on any of those documents earlier, he  
19 said no. So I'm objecting to that, and I would move to  
20 strike any testimony at the trial related to that.

21 MR. KEMNA: I'll just note for the  
22 record that a list of every document contained within the  
23 group Exhibit No. 1 was provided to plaintiff's counsel in  
24 advance of this deposition to be responsive to their  
25 request for production of documents in this case, and Dr.

1 Giannini has stated that he has reviewed these documents  
2 and is prepared to testify within his area of expertise  
3 regarding these documents at trial.


4 MR. HOAG: As I said, there's nothing  
5 in the disclosure statement that says he's going to talk  
6 about the history of the tobacco companies' research and  
7 whether or not that research would have contributed in any  
8 way to the overall research that was available at the time;  
9 and to the extent he plans to testify about that, I would  
10 move to strike.

11 And again, I'm finished. And I'm ordering a  
12 Minuscript, a copy of the deposition and a disk.

13 MR. KEMNA: For the record, we do not  
14 waive signature so that Dr. Giannini will require a copy of  
15 the transcript for his review and whatever appropriate  
16 corrections. I think that's it.

## REPORTER'S CERTIFICATE

I HEREBY CERTIFY that the above and foregoing is a true and correct transcript of all the testimony introduced and proceedings had in the taking of the testimony in the above-entitled matter, as shown by my stenotype notes taken by me at the time said testimony was taken.

  
Lisa C. Nagy-Baker  
Registered Diplomat Reporter

1 STATE OF OHIO )  
 2 MAHONING COUNTY ) SS: CERTIFICATE

3 I, DR. JAMES GIANNINI, depose and say  
 4 that I have read the foregoing deposition and find it true  
 5 and correct, unless otherwise specifically excepted to and  
 6 indicated on Page 73-A, and any following numbered pages  
 7 thereafter, if applicable, and I subscribe my signature to  
 8 the aforesaid deposition this \_\_\_\_\_ Day of \_\_\_\_\_,  
 9 1998.

10  
 11 DR. JAMES GIANNINI

12  
 13 Before me, a Notary Public within and  
 14 for the State of Ohio, personally appeared DR. JAMES  
 15 GIANNINI, who, being first duly sworn, deposes and says  
 16 that he has read the foregoing deposition and finds it true  
 17 and correct to the best of his knowledge, information and  
 18 belief, unless otherwise specifically excepted to and  
 19 indicated on Page 73-A, and any following numbered pages  
 20 thereafter, if applicable.

21 SWORN AND SUBSCRIBED before me this  
 22 \_\_\_\_\_ Day of \_\_\_\_\_, 1998.

23  
 24 Notary Public  
 25 My Commission Expires

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HUMPHREY

JOSEPH H. HUNT

TOBACCO INDUSTRY RESEARCH COMMITTEE  
150 EAST FORTY-SECOND STREET  
NEW YORK 17, N.Y.

March 7, 1963

KING & SPALDING  
101 PEACHTREE STREET  
ATLANTA, GEORGIA 30303-1763  
FACSIMILE 404/572-5100  
404/572-3530

Dr. V. Hamill, M.D.  
Staff Medical Coordinator  
Surgeon General's Advisory Committee  
on Smoking and Health  
National Library of Medicine  
Bethesda 14, Maryland

Dear Doctor Hamill:

Some time ago, when you and Mr. Roos were in New York, you indicated that you would like to have some material on the Possible Beneficial Effects of Tobacco.

I am sending herewith a list of references for study in this connection. Under separate cover I am mailing seventy-five additional copies.

This material is not sent to present a case for tobacco or to initiate or support argument or controversy. It merely suggests some topics that indicate the possible beneficial effects of tobacco on certain people under certain physical, mental or emotional conditions. These possible effects may be of interest for further study.

There may well be other topics which we have overlooked because we have never made an organized effort to cover this field completely.

With kindest personal regards.

Sincerely,

*Robert C. Hockett*

Robert C. Hockett, Ph.D.  
Associate Scientific Director

RCH:ek

encl.

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S-36

TIRC  
March 11, 1963POSSIBLE BENEFICIAL EFFECTS OF TOBACCO

In considering the material, it is well to remember that the response to smoking is an individual matter. Broad generalizations of any sort concerning any possible effects of smoking are therefore scientifically unwise and may be misleading.

No effort has been made to indicate any relative order of importance in their presentation.

1. Lower blood cholesterol. (Hunter & Wong 1961) (Miller et al. 1958)  
(Bergste-Stewart 1961)
2. Digestion. (Lancet 1910)
3. Laxative Effects. (Dickson & Wilson 1924) (Dixon 1927)  
(Johnson 1929) (Schneider & Ivy 1939) (Calatayud 1944)
4. Post-operative gastrointestinal atony. (Körner 1961)
5. Increased bile secretion. (Liscia 1954)
6. Thiamine retention. (Strauss & Scheer 1939)
7. Ascorbic acid excretion. (Vesulet 1954, 1956)
8. Increase of certain blood elements. (Eisen & Hammond 1956)  
(Blackburn et al. 1960) (Von Kreuziger 1956) (Baronchelli 1952)
9. Increased adrenal-medullary function. (Larson et al. 1961)  
(Silvette et al. 1961)
10. Increased blood sugar. (Larson et al. 1961) (Wachholder 1948)  
(Blackburn et al. 1960) (Anand et al. 1962)
11. Suppression of aphthous ulcers. (Bookman 1960)
12. Increased resistance to infections. (Frei 1941) (Boake 1958)  
(Silvette et al. 1958) (Cavallaro 1910) (Fullerton 1912)  
(Schmidt 1939) (Appleton 1928)
13. Increase of antidiuresis. (Larson et al. 1960) (Mayer 1957)  
(Moretti et al. 1957)

14. Tranquilizing effects. (Silvette et al. 1958) (Larson et al. 1961)  
(Wiggers 1960)
15. Effects on obesity. (Larson et al. 1961) (Passey et al. 1961)  
(Blackburn et al. 1960) (Berkson et al. 1960) (Stamler et al. 1961)  
(Ashford et al. 1961) (McCann et al. 1961) (Damon 1961, 1962)  
(Rosenberg 1959) (Brozek & Keys 1957)
16. Acceleration of labor. (Burn et al. 1945)
17. Improvement of night vision. (Troemel et al. 1951) (Bohne 1962)
18. Increased clot-dissolving activity. (Bronte-Stewart 1961) (Bellet et  
al. personal communication 1962)
19. Increased expectorant action. (Barsch et al. 1952) (Leese 1956)
20. Increased adrenocortical function. (Hökfelt 1961)
21. Serotonin metabolism. (Schlüsselwein et al. 1961, 1962) (Larson et al.  
1961:69-70)
22. Effects on fatigue. (Fischer et al. 1960) (Raphael 1920) (Hull 1924)  
(Simonson 1959) (Larson et al. 1961:573) (Chapman 1944)
23. Oral stratification. (Madathur 1958)
24. Rise in serum free fatty acids. (Kershbaum et al. 1960, 1961, 1962)
25. Effect on cardiovascular function. (Larson et al. 1961:192)  
(Bargeron et al. 1956, 1957) (Regan, Kellems & Bing 1960)

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# TOBACCO

## Experimental and Clinical Studies

*A Comprehensive Account of the World Literature*

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## ABSORPTION AND FATE

### ABSORPTION

In this section, the absorption of nicotine and other products of tobacco use or combustion will be treated in a non-quantitative, but, whenever possible, comparative manner. For a discussion of the rate of absorption of nicotine and other tobacco products, the amount of absorption, and factors influencing both, see the sections below.

#### ARTS

##### Oral Cavity

Early workers reported that nicotine placed on the tongue of various species of animals caused death with characteristic signs of nicotine poisoning (Orfila, 1851a; Sandencorput, 1851, 1852; among others). Superficial necrosis of the oral mucosa of cats, produced by scalding or by freezing, retarded absorption or penetration of nicotine (Macht, 1933). Walton (1944) considered that the fat-water distribution coefficient was the most important determining factor in absorption of drugs from the sublingual space: the higher this coefficient, the greater should be the predicted absorbability. Nicotine, however, was something of an exception to this correlation, since its fat-water distribution coefficient is low (2.6); but, since it is completely miscible with fat in all proportions, its behavior may thus be considered more as a modification of the correlation than as a contradiction of it. To state the correlation more completely, then, it might be said that the penetrability of drugs through the oral mucosa was favored by a high fat-water distribution coefficient or by an exceptionally high fat solubility. In addition to the unique solubility of nicotine in fats, this alkaloid is unusually alkaline and irritant; the latter factor was possibly an important conditioning one in its high degree of penetrability.

Nicotine is readily absorbed from chewing-tobacco, although the absorption is less rapid than occurs on smoking with inhalation (Gaede, 1941a). Nicotine was found to be absorbed from tobacco-smoke even when the smoker did not inhale (K. B. von Lehmann, 1906). Whether or not the carbon monoxide in tobacco-smoke could also be absorbed through the mucous membranes of the mouth was left undecided (Jongbloed, 1939).

##### Respiratory Tract

Injection of 1 ml. of 1:1000 per kg. nicotine into the nasal cavity of dogs anesthetized with chloralose resulted after 25-30 seconds in increases in respiration, blood pressure, and pulse; in contrast to animals given the same dose intratracheally, intra-nasal injection was not fatal (Dobrzanski, 1926). Macht (1937-38, 1938) reported rapid absorption of nicotine from the nose of rats, guinea pigs, and rabbits. Gaede (1944) estimated the effectiveness of nasal vs. intravenous doses of nicotine at about 1:10; he used nicotine alkaloid

and nicotine hydrochloride in rabbits, cats, and dogs, the species effect of nasal administration diminishing in that order.

Comel (1936) reported that he had never observed absorption of the dust particles by the nasal mucosa following experimental insufflation of snuff in guinea pigs.

Macht (1933, 1937-38, 1938) found that the absorption of nicotine applied to the pharynx of cats and rabbits was retarded when the pharyngeal mucous membrane was severely injured (i.e. more or less necrosed), while the alkaloid penetrated readily when the chemical reagent was not a destructive one. When applied to the pharynx, epinephrine and ephedrine did not retard penetration of nicotine; zinc sulphate, glycerites of tannin, and mercuric chloride delayed absorption; mercuricbrome and merodicein and aluminum acetate effected no change in the absorption time of nicotine, while gentian violet delayed it (Macht, 1933). Damage by freezing with dry ice, by scalding, and by other physical agents also interfered with the absorption of nicotine (Macht, 1937-38, 1938).

Nicotine (1 ml. 1:1000 solution per kg.) introduced into the trachea of chloralosed dogs was absorbed, and was followed in 10-20 seconds by increases in respiration, blood pressure, and pulse, and ultimately by death (Dobrzanski, 1926). In dogs fitted with tracheal cannula, cigarette-smoke directed into the lungs gave a greater hypertensive response than directing the smoke into the upper respiratory passages, while in the rabbit, inhalation of cigarette-smoke caused marked respiratory and cardiovascular effects, but when the smoke was simply circulated through the mouth, these effects were greatly reduced (Jourdan and Collet, 1944, 1950).

H. Keller (1935) incorporated 3 mg. of nicotine in 10 ml. of 2.5% Protosil-Soluble, and had subjects inhale the solution by means of an atomizer. Rises in blood pressure and pulse rates were evidence of pulmonary absorption of nicotine.

Ermals and Holsti (1955) studied the rate of absorption of tobacco-tar in the organs of the respiratory tract of laboratory animals, but, owing to difficulties in technique and interpretation of data, a detailed description of this portion of the study was not reported.

A number of cases have been reported of nicotine poisoning caused by the inhalation of nicotine sprays. For example, a woman spraying flowers in an enclosed place with a preparation containing nicotine developed mild nicotine poisoning; there was no contact whatsoever of the solution with her hands or body, and the poisoning was attributed to inhalation of nicotine (Gindhart, 1939). A man inhaled some nicotine sulphate while treating cattle, and developed severe nicotine poisoning (Pybus, 1943). Other cases are mentioned or described in Chapter 14.

##### Gastro-intestinal Tract

Travell (1940a) reported that fatal absorption of nicotine took place from the ligated stomach of the cat when the pH of the gastric juice was alkaline, but not when it was acid.

Strong infusion of tobacco injected into the rectum killed 3 dogs and 1 cat in 7-10 minutes (B. C. Brodie, 1811). In man, numerous cases of nicotine poisoning have been recorded as a result of the therapeutic use of tobacco-infusion enemas (Acta Helvetica, 1762; "Physician," 1813; Paris and Fonblanque, 1823; Ansiaux, 1827; Grahl, 1830; Tavignot, 1840, 1841; Bertini, 1846; Eade, 1849; Copland, 1858; J. B. Wilkinson, 1889; Lancet, Lond. 2: 765, 1894; Ehrnrooth, 1912; Fulpius, 1913; Garvin, 1913; Detis, 1937; Willis, 1937; among others); absorption from this route is so rapid as to be extremely dangerous. According to S. Wright (1846), a man sat over a chamber-pot containing half an ounce of tobacco, together with burning coals, to ease himself of piles; in a few minutes, he fell prostrate with characteristic symptoms of nicotine poisoning. Gill (1901) reported that an ounce of cut Cavendish tobacco inserted into the rectum by a man desiring to smuggle the tobacco into a prison also produced nicotine poisoning.

Fujita (1925, 1927b) applied nicotine to the mucous surface of isolated intestine, and noted almost no effect, indicating that at this surface the drug penetrated the tissue poorly. The action of nicotine on the Magnus preparation he thought to be due to contact with the serous surface. Shioya (1927, 1929) applied drugs, among which was nicotine, to either the serous or mucous surface of isolated rabbit intestine, or else administered them via its vascular supply. In general, drugs brought in contact with the mucous surface acted more weakly than when applied to the serous surface or administered via the vessels, from which this author concluded that penetration of the drugs through the mucous surface was more difficult. In this connection, Terasaka (1927, 1929) found that application of nicotine to the serous surface of excised rabbit uterus produced more intense response than did application to the mucous surface.

#### Skin

From his studies, McIndoo (1916) concluded that nicotine spray solutions do not penetrate the integuments of insects. However, tarsi of the cockroach and locust were found to be permeable to nicotine vapor (C. H. Richardson, Glover and Ellnor, 1934); this was also true for the imagoes and older juvenile stages of the cockroach and of the larvae of the nocturnal moth *Heliothis obsoleta* Fab. (Glover, 1936).

Commercial (92-95%) nicotine applied to the underside of the wings and tail feathers of chickens resulted in acute nicotine poisoning (C. Dickson, 1943).

According to Haag and Neale (1943), nicotine alkaloid is absorbed through the skin of the tail in white mice. The rate of absorption varied largely with the concentration of nicotine in the solution, but nicotine salts were practically not absorbed through the intact skin of the tail.

Nicotine alkaloid applied to the shaven skin of rats and guinea pigs was readily absorbed, and produced toxic symptoms (Pfeiffer, 1937).

Randolph and Dixon (1854) placed nicotine on an adhesive plaster, and applied it to the abdominal skin of rabbits. In one case, 1 drop of nicotine caused death in 5 hours 11 minutes; in each of 3 cases, application of 10 drops was fatal in 109 minutes, 28 minutes, and 36 minutes, respectively; in a fifth case, 15 drops caused death in 28 minutes. Burstein (1927b) tested the absorption of nicotine from dry tobacco dust deposited on the shaven skin of rabbits. The animals were then wrapped in cloth, but no symptoms were observed, which might indicate that the amount of nicotine absorbed was too small to cause symptoms. Manganaro (1935) applied nicotine base, nicotine tartrate, and tobacco extracts to large areas of

the skin of the abdomen of rabbits and dogs following removal of hair, and found evidence that nicotine base was rapidly absorbed, whereas nicotine tartrate was not absorbed, or only very slightly so. Absorption of nicotine occurred from the tobacco extracts, the rapidity and degree being proportional to the concentration of the alkaloid in the extract.

Faulkner (1933) applied 2-10 ml. amounts of "Nico-Fume Liquid" (a 40% solution of free nicotine) to 5-7 cm. areas on the abdomen of 5 cats; the area was then covered by an inverted glass dish held down tightly with adhesive. All the animals died of nicotine poisoning, while 3 cats similarly treated with "Black Leaf 40" (40% nicotine sulphate) showed no effects.

Willenbücher (1940) found that nicotine was absorbed through the shaved skin of dogs; significant variations between animals were noted.

André (1941) reported that of 36 horses washed with a solution of nicotine (24-25 ml. of nicotine per horse) for the purpose of delousing, 8 developed typical symptoms of nicotine poisoning. Characteristic nicotine poisoning, sometimes fatal, has also been reported to follow dressing cattle with nicotine solutions (de Leur, 1934; Kamarrás, 1936), solutions of nicotine sulphate (Hornby and French, 1942; T. H. Jones and John, 1943; Pybus, 1943; McGrath and Campbell, 1944), or tobacco extract (R. B. H. Murray, 1943).

Sufficient absorption of nicotine to cause serious or fatal poisoning in humans has resulted from application to the skin of tobacco or tobacco leaves (B. J. A. Murray, 1793; W. A. G., 1840; Polko, 1854; J. G. Stephenson, 1857; Namias, 1864; Tosini, 1864; Allan, 1871; O'Neill, 1879; Deacon, 1926; among others), tobacco infusions or decoctions (S. Jackson, 1826; Sigmond, 1838a; Blanchard, 1869; Auché, 1891; Jausion et al., 1940), or "tobacco tars" (often called pipe-oil, empyreumatic oil of tobacco, pipe-residue) (Calosi, 1858; Turcibetti, 1860; Boston M. & S. J. 79: 340, 1868; M. G. Evans, 1869; Hare, 1885; J. O. Jones and Morris, 1926; among others). In these cases, the skin was normal, wounded, ulcerated, scorbutic, or showing chronic eruption (review of a series of early cases by Gallavardin, 1864a, b). It is interesting to note that, although B. J. A. Murray (1793) reported that a salve made of snuff and butter rubbed on the heads of 3 children to cure head lice resulted in nicotine poisoning, Somervail (1838) noted that snuff plasters applied to the skin did not produce symptoms of nicotine poisoning unless the cuticle were abraded. It may be noted the Milev (1954) stated that abrasions of the skin acted as sites of absorption in tobacco-workers harvesting tobacco leaves moist with dew. The ready penetration of nicotine through the intact skin is evident from an account by Foville cited by Ritchie (1926). Foville (*Influence des Vêtements sur nos Organes*, Paris, 1834) described the French military head-gear of his time as especially tight, heavy, and unventilated; and he added that soldiers were accustomed to carry their tobacco inside their caps, and that symptoms of narcotism often resulted. A number of cases of nicotine poisoning resulting from contact of the skin with nicotine solutions, as in gardeners using nicotine sprays, have been reported (D. J. B. Wilson, 1930; Lockhart, 1933; Jungerhaus, 1937; Wehrin, 1938; Gindhart, 1939; Mattei, 1939; Beremer, 1948); absorption in this manner appears to be very rapid. The form of the preparation is not without influence: nicotine salicylate made into a 0.1% salve with lanolin did not cause any symptoms of poisoning upon inunction, while treatment with nicotine soap caused marked intoxication symptoms in 2 of 8 patients (Wolters, 1898). These differences are undoubtedly related to the amounts of the free alkaloid.

### Mucous Membranes

Nicotine was absorbed less efficiently through mucous surfaces than through serous surfaces (Fujita, 1925, 1927; Shioya, 1927, 1929; Terazaka, 1927, 1929). Nicotine penetrated more rapidly through normal mucous membranes than through pathological or necrotic mucosa, unless there were open bleeding vessels (Macht, 1933, 1937-38, 1938).

### Pericardium

Baltaceanu and co-workers (1936) introduced nicotine into the pericardial cavity of dogs under chloralose anesthesia with artificial respiration and the thoracic cavity opened. Within a few seconds, marked increases in arterial pressure and cardiac volume were observed, and 3-8 minutes after penetration of nicotine in the pericardium, the cardiac effects were extreme.

### Eye

One drop of nicotine applied to the eye of a dog caused severe poisoning with recovery (Orfila, 1851a), and, in another animal, death in 2-3 minutes (Orfila, 1851b, c). The systemic effects of nicotine were also produced in the rabbit by placing a small drop of nicotine in the eye (Sturninsky, 1869). Nicotine also was rapidly absorbed from the conjunctiva of rats and guinea pigs as well as of rabbits (Macht, 1937-38, 1938). Nicotine poisoning resulted following introduction of the drug into the conjunctival sac of dogs, whether the nasal duct were patent or occluded (Macht, 1938). Absorption from the conjunctiva in rabbits, cats, and dogs was somewhat more rapid than from the nasal cavity (Gilde, 1944).

### Genous System

Application of little discs of blotting paper saturated with nicotine to the cerebral cortex motor area, or on the posterior surface of the bulb, of dogs resulted after 20-40 seconds in nicotine dyspnea (Baglioni, 1927).

Nicotine in doses of 1-2 mg. injected into the cisterna of dogs was rapidly absorbed from the cerebrospinal fluid into the blood; onset of blood-pressure rise was nearly as rapid, and magnitude nearly as great, as if the injection had been made intravenously (Dixon and Halliburton, 1915-16). Nicotine in amount of 1 mg. by sub-occipital puncture produced characteristic cardiovascular, respiratory, and emetic effects (Mergier and Delphaut, 1936).

Funakoshi and Ogata (1930) exposed the sciatic nerve of rats, and by means of a fine needle inserted centrally under the nerve sheath a solution of nicotine dissolved in machine oil. Convulsions and other signs of nicotine intoxication resulted. Barry (1934) reported that nicotine injected into the vagus trunks of dogs or rabbits caused the same train of symptoms as if the drug were injected intravenously. These results were evidently due to systemic absorption of the nicotine, however, for when the experiment was repeated with the vagus trunk doubly ligated to occlude the lymphatics and blood vessels of the sheath without injury to the nerve fibers, injection of nicotine into the sheath between the ligatures had only the local action of blocked conduction (Barry, 1935a).

### Genito-urinary Tract

Nicotine was rapidly absorbed from the vagina of rats, guinea pigs, and rabbits (Macht, 1937-38, 1938). Nicotine was also readily absorbed through the vaginal mucous membrane of white mice (Haag and Neale, personal communication; also 1940). Filling the vaginal canal with a 2% solution

of nicotine in saline produced death in the majority of the mice employed. Nicotine sulphate was also apparently absorbed through the vaginal mucosa, but to a lesser extent or less rapidly than nicotine, since the concentration of nicotine as nicotine necessary to produce death in a comparable number of mice was about 10 times that for the free nicotine solution.

Tobacco injections per vaginam in a patient with tetanus resulted in characteristic symptoms of nicotine poisoning (Smart, 1830).

Application of nicotine to the serous surface of excised rabbit uterus produced more intense response than did application to the mucous surface; the difference was attributed to differences in absorption capacity of tissues and penetrability of the drugs used, as well as their mode of action (Terazaka, 1927, 1929).

Macht (1937-38, 1938) reported that nicotine was rapidly absorbed from the urethra of rats, guinea pigs, rabbits, and dogs, but not from the bladder of dogs (Macht, 1916).

Travell (1940b) injected buffered solutions of nicotine into the bladder of cats, and found the absorption and toxic effects progressively increased from pH 4.5-6.0 to pH 9. The volume of the injected solution remained constant, while the nicotine was absorbed. Comparing nicotine absorption from the bladder with that from the stomach, (Travell, 1940a), the author concluded that the rates of absorption were approximately equal at equal pH, when the greater surface of the stomach was taken into consideration. Travell, Bodansky and Gold (1940) suggested that when the urine was alkalinized, the cat might be poisoned by the nicotine which had collected in its own bladder and which in consequence of the alkalinization was now reabsorbed, and that a similar poisoning might occur in tobacco-smokers from reabsorption of nicotine from the human bladder when the urine was alkaline. This latter suggestion has been rebutted by Haag and Larson (1942).

### Subcutaneous

Nicotine deposited through an incision beneath the skin in dogs and cats produced characteristic poisoning (Longchamps, des Loiseleur and Melier, 1844-45). Tobacco-smoke drawn into a syringe and insufflated under the skin of white mice caused toxic symptoms and even death, while insufflation of air or smoke from hay had no ill effect (Guillain and Gy, 1907a). The case was reported of a man who suffered a deep laceration of the tip of the finger into which a combination of sulphur and nicotine was driven; typical acute nicotine poisoning resulted (Gindhart, 1839).

Travell (1940b) found that, upon subcutaneous injection of nicotine into the inguinal fold in rats, both nicotine and fluid were rapidly absorbed. Comparatively, absorption of nicotine from the subcutaneous tissues was greater than from the urinary bladder or the ligated stomach.

### Miscellaneous

Underhill (1905-06) reported that painting the pancreas with solutions of nicotine and piperidine caused hyperglycemia in dogs. However, since painting the spleen with piperidine was also effective, pancreatic absorption must not have been specifically involved.

F. R. Chapman (1880) reported that a plug of tobacco the size of a pea inserted into the hollow of a painful tooth resulted in characteristic nicotine poisoning.

Instillation of a few drops of nicotine into the auditory canal of cats and dogs was fatal in 15-20 minutes (Macht, 1916, 1924). Nicotine was also rapidly absorbed from the

intact ear drum of rats, guinea pigs, and rabbits (Macht, 1937-38, 1938).

Using blood-pressure changes as an index of the effect of absorption, Childrey and Essex (1931) found that 1 mg. of nicotine injected intravenously in dogs resulted in a marked rise in blood pressure; 20 mg. injected subcutaneously into the same animal caused an immediate slight rise in blood pressure lasting 7 minutes, but 20 mg. injected into the left frontal sinus was without effect during a period of 35 minutes. The same negative result followed an injection of 80 mg. nicotine into the sinus of another dog and one of 4 mg. into the sinus of a cat.

According to Portier (1930a), 2% or 10% nicotine applied to one or both of the antennae of butterflies for 1 minute or less resulted in nicotine intoxication. If, after having impregnated the club of one antenna with nicotine solution for 1 minute, it was immediately severed, the intoxication came on normally or even more rapidly than if the antenna were intact. By measuring the length of the antenna and the latent period of intoxication, Portier calculated that the transmission of the poison occurred at the rate of about 1 meter per hour.

#### RETENTION OF TOBACCO-SMOKE CONSTITUENTS IN MAN

W. A. Wolf, Purdom and Isenbarger (1954b) introduced smoke from cigarettes which had been treated with radioactive isotopes ( $K^{42}$  and  $Na^{24}$ ) into the respiratory tract of anesthetized dogs under artificial respiration. The amount of radioactive material present in the lung at the end of the smoking period was calculated to be only 6-25% of the total amount deposited in the lung. Later, Wolf (1955) administered cigarette-smoke to some 60 dogs under thiopental and artificial respiration in such manner that the animals received on a per kg. body weight basis and per minute of smoking time a dose which could be related to the human smoker. Three dose levels were used: the pack-a-day level, the heavy chain-smoker level, and an acute experimental dose which was 6 times the chain-smoker dose. At the end of the smoking period, of 15, 30, or 60 minutes later, the animals were sacrificed, the lungs removed, and their nicotine content determined colorimetrically. Results of individual experiments showed a wide variation, with considerable overlapping of the dose levels; but, as a general trend, in tests with the pack-a-day and chain-smoker dose levels, 90-95% of the nicotine dose disappeared from the lungs during the smoking period. One hour after the end of the smoking period, the lungs were either nicotine-free or contained only 1-2% of the total dose. In the experiments with the acute experimental dose, some 75-95% of the nicotine disappeared from the lungs during the smoking period; and during the hour following, the nicotine content of the lungs fell rapidly to about the same level found in these tests with the human-smoker dose of cigarette-smoke.

Larson and Harlow (1958) reported experiments in which  $C^{14}$ -randomly-labeled glucose was added to the tobacco in cigarettes, and the puffed smoke deposited in the respiratory tract of dogs. Tissue distribution of  $C^{14}$  and its content in the various excreta (including expired air) were determined at 4 minutes and 2 and 24 hours following inhalation of the smoke. The data at 4 minutes showed that only about 7% of the  $C^{14}$  found was still in the respiratory tract, the remainder having been absorbed into the other body tissues, and that excretion had begun. Thereafter, a residual fraction was more slowly absorbed. The data at 2 and 24 hours indicated that elimina-

tion of absorbed carbon via expired air and urine was rapid during the first few hours, then slowed, leaving about one-third still in the body at 24 hours. The authors felt that some of this remainder had entered into normal metabolic pathways in the body.

Holland *et al.* (1958) described a machine for exposing rabbits to cigarette-smoke inhalation. Radioactive  $As^{74}$  was used to establish the fact that the rabbits did inhale the smoke into their lungs. In addition to detailed studies on the distribution of inhaled arsenic along the respiratory tract (see Chapter 7), certain rabbits following inhalation of smoke were measured for radioactivity over marked areas along the nasal passages, larynx, trachea, and lung fields. Using the original uptake as 100%, the radioactivity fell off rather rapidly during the first two days, and then tapered off slowly.

#### Nicotine

Biederbeck (1908) studied the absorption of nicotine vapors from the oral cavity. He introduced 22-28 mg. of nicotine into his mouth, and failed to find any nicotine in the expired air in 3 experiments, whereas in 2 other tests, about 2 mg. nicotine were found, corresponding to an absorption of 90-94%. A repeat experiment by a medical student gave similar results, and thus confirmed earlier experiments by J. Willke, also working under the direction of K. B. von Lehmann, who found 68-100% absorption of nicotine vapors from the mouth. Lehmann (1909) himself reported that when pure nicotine vapors were drawn into the mouth, the nicotine was absorbed almost completely, or at least up to 90%.

#### Nicotine in Cigarette-Smoke

In experiments with cigarette-smoke, Biederbeck (1905) found that after simple puffing without inhalation, only about 20% of the nicotine in the main-smoke stream was absorbed, from which he concluded that the absorption of nicotine in the human mouth was much poorer from smoke than from air. Lehmann (1909) considered that there was something about tobacco-smoke which interfered with the absorption of nicotine.

Lehmann (1905, 1909) and his students studied the absorption of nicotine by the smoker by two methods: a direct one, in which the mouth cavity was rinsed out repeatedly during smoking, and the nicotine in the rinse-water determined; and an indirect method in which the main-stream smoke and the exhaled smoke were analyzed for nicotine and the absorption obtained by subtraction. The first method gave results which were too high; the second was considered to be the more accurate method. By the direct method, it was calculated that 3.5-6.8 mg. nicotine were absorbed per gm. of cigar and 1.4-1.7 mg. per gram of cigarette smoked, apparently without inhalation. By the method of differences, it was calculated that 1.7-2.5 mg. nicotine were absorbed per gram of cigar, and 0.8-1.5 mg. per gram of cigarette smoked (Lehmann, 1908). When a cigarette was smoked down to a minimum stub, an absorption of about 10% of the total nicotine was found (Lehmann, 1909). In contrast to this small absorption of nicotine from the main-stream smoke, pure nicotine vapors when drawn into the mouth were absorbed to the extent of 90-94% (Biederbeck, 1908; Lehmann, 1909).

Baumberger (1923b) calculated that, of the nicotine in cigarette-tobacco, about 14-33% appeared in the smoke puffed. In puffing, an average of 66.7% of the smoke was retained in the body of the subject, and in inhaling, an average



of 88.2% was retained (Baumberger, 1923c). From these figures, Baumberger (1923b) calculated that about 66.7% of the nicotine entering the mouth in puffing would be retained, and 85% in inhaling. It was suggested, however, that probably a large proportion of the smoke retained in the body was removed by expectoration before absorption of nicotine had been completed (Baumberger, 1923b, c). In mouth smoking, only about 2% of the nicotine contained in the smoke was found by Wenusch (1927-28) to be absorbed; in the case of ordinary inhalation, the figure was 50%; with excessive inhalation, 85%.

Burstein and Goldenberg (1928) calculated that a subject who smoked 5 cigarettes in 25 minutes absorbed 3.2 mg. nicotine, or 0.06 mg. per kg. body weight. Another subject, who smoked 7 cigarettes in 45 minutes, was calculated to have absorbed 4.48 mg. or 0.07 mg. per kg.

A. Winterstein and Aronson (1925) determined the amount of nicotine absorbed by the smoker by collecting the smoke exhaled and subtracting the nicotine exhaled from that known to be in the main-stream smoke. In smoking without inhaling, only 2.5-4.7% of the total nicotine in the consumed tobacco was absorbed when light tobaccos were used, 5.5-7% when dark tobaccos were smoked, and 11.7% with a cigarette made from cigar-tobacco. When inhaling, the amount of nicotine absorbed was 3 times greater if the smoke were inhaled in 2 seconds when held for a long time, all the nicotine in the smoke was absorbed.

Bodnar, Nagy and Dickmann (1935) studied the absorption of nicotine from cigarette-smoke by subtracting from the known nicotine content of the cigarette the amount of nicotine lost in the side-stream smoke and that in the exhaled smoke plus that left in the cigarette holder (the cigarette being apparently completely smoked); in these calculations, they assumed that no nicotine was destroyed in the burning of the cigarette, which is now known to be a false assumption. Using this method, these authors found 60% retention of nicotine on puffing, and over 90% on inhaling. Pyriki (1943) pointed out that his results with non-inhalation (Pyriki, 1932b) differed markedly from those obtained by Bodnar and his co-workers.

I. H. Pierce (1937, 1941) made analyses of the tobacco, the stubs, the smoke passing off from the burning tip, and the smoke drawn into the mouth, both before and after inhalation, and designated the difference between the latter as nicotine absorbed. By this method, 78.5% of the nicotine was retained on puffing, 85% on deep inhalation.

According to Hillsman (cited by Haag and Larson, 1944), in the non-inhaler, only a small fraction of the 3 mg. nicotine in main-stream smoke was retained, amounting to approximately 12%.

An entirely different method was employed by Chalmers and Lewis (1933) to estimate the amount of nicotine absorbed in smoking. Comparison of the antidiuretic effect of smoking with that of nicotine infusions suggested to these authors that the smoker derived 1.0-1.5 mg. nicotine from 1 cigarette, provided he inhaled vigorously.

Greenberg, Lester and Haggard (1952) studied the effect of moisture on the transfer of nicotine to the main-stream smoke and its retention on smoking. Smoking dry cigarettes without inhalation resulted in nicotine retentions of 26% and 45%; smoking moist (11% moisture content) cigarettes, 8% and 16%. Smoking either dry or moist cigarettes with inhalation resulted in nicotine retentions of 96-98%.

A summary of the percentage absorption of nicotine in

TABLE 1-1  
Absorption of Nicotine in Cigarette Smoking

Author	Per Cent Absorption of Nicotine from Cigarette Main-Stream Smoke on:	
	Puffing	Inhaling
Biederbeck, 1908	ca. 27	
K. B. Lehmann, 1909	ca. 10	ca. 80
Baumberger, 1923b	66.7	88
Heinz (1923)	17.5	87.6
Wenusch, 1927-28	8.3	80-85
Winterstein and Aronson, 1925	2.5-11.7	7-35 (2 sec.)
		100 (prolonged)
Pyriki, 1932b	4-5	84-95
Bodnar, Nagy and Dickmann, 1935	60	93
Pierce, 1937, 1941	78.5	95 (deep)
Wenusch, 1942a, b	5-10	50 (moderate)
		65-100 (excessive)
Pyriki, 1943	5	66 (weak)
		66-76 (moderate)
		84-95 (deep)
Hillsman, cited by Haag and Larson, 1944	ca. 12	ca. 90
J. A. M. A. 150: 825, 1946	67	88
Laskowski, 1951		67
Greenberg, Lester and Haggard, 1952	6-45	96-98

\* These appear to be the same figures as those of Baumberger (1923b).

cigarette-smoking found by the above and other authors is given in Table 1-1.

#### Nicotine in Cigar-Smoke

According to Winterstein and Aronson (1925), the amount of nicotine absorbed by the smoker from cigars without inhalation was 13-20% of the total nicotine in the tobacco smoked. These authors concluded that more nicotine was absorbed from cigar-smoke, even without inhaling, than from cigarette-smoke.

Greenberg and co-workers (1952) reported that smoking a dry cigar without inhalation resulted in 16% nicotine retention by the body, whereas similarly smoking a moist (11% moisture content) cigar resulted in only 4% nicotine retention. When either a dry or moist cigar was inhaled, the nicotine retention rose to 96-97%.

Wenusch (1942a, b) stated that nicotine retention from cigar-smoke was harder to estimate than that from cigarette-smoke, due to varying alkalinity of cigar-smoke and varying degree of filtration through the unsmoked portion. A cigar with 1% nicotine and with strongly alkaline smoke deposited 3 times as much nicotine in the mouth as a similar cigar with only a weakly alkaline main-stream smoke; and from a cigar 121 mm. in length, 0.2 mg. nicotine was deposited in the mouth from the first 37 mm., 1 mg. from the second 37 mm., and 3.1 mg. from the third 37 mm. Wenusch concluded that in cigarette-smoking, the amount of nicotine absorbed depended on the degree of inhalation, whereas with cigar-smoking, it depended on the alkalinity of the main-stream smoke.

and the length of the unsmoked stump. Assuming that 30 cigarettes weighing about 1 gm. apiece and containing about 2% nicotine in the tobacco might contain just about the same amount of nicotine as 6 cigars weighing about 5 gm. each and with the same nicotine content; and assuming one-fourth of the nicotine content of the tobacco to appear in the puffed smoke, it was said to be possible for the 30 cigarettes inhaled to yield about the same amount of nicotine absorption as the 6 cigars not inhaled (*J. A. M. A.* 130: 825, 1946).

The subject of the absorption of nicotine by the smoker has been reviewed in part by Pyriki (1937).

#### Nicotine in Chewing Tobacco

Gaede (1941a) studied nicotine absorption from chewing tobacco. A chew of 0.6 gm. contained on the average 15 mg. nicotine. After 1 hour's chewing, the nicotine content of the residue was reduced by 33%; at 2 hours, by 50%; at 4 hours, by 60%; and at 8 hours, by 90%. This was said to be a less rapid absorption, even in the early period, than occurred on smoking with inhalation.

In 21 habitual tobacco-users who chewed 15.6-58.4 gm. tobacco during 6.5-8 hours, W. A. Wolf and Giles (1950) found the amount of nicotine absorbed to be 8.0-87.7 mg.

#### Nicotine in Snuff

Gaede (1944) stated that the daily dose of nicotine obtained by the chaser [sic] of snuff was from 20-60 mg.

#### Nicotine from Inhalation of Tobacco Dust

Burstein (1927a, b) reported that the average amount of tobacco dust inhaled by a workman in a tobacco factory varied from 2.8 to 17.3 mg. per hour. Since the average nicotine content of tobacco dust was found to be 1.73%, the total amount of nicotine retained by the body from dust inhaled was calculated as 0.305-1.903 mg. per 8-hour day. Other calculations (based on distribution data cited from Lehmann, Saito and Gfrorer, *Arch. Hyg., Münch.* 75: 152, 1912) led to the conclusion that 1 mg. nicotine was retained for every 60 mg. of dust inhaled, and that, of the inhaled tobacco dust, approximately 6% was breathed out, 28% retained in the nose, 22% penetrated into the lungs, and 44% capable of reaching the gastro-intestinal tract.

TABLE 1-2

Retention of Cigarette-Smoke Constituents by Smokers  
(Adapted from Laskowski, 1951)

Substance	Per Cent Retained in Lungs
Total amount of tar substances	37
Substances insol. in ether	14.9
Free bases	25.9
Aldehydes and ketones	99.0
Free carboxylic acids	44.0
Phenols	57.0
Neutral substances	30.8
Unaccounted and inorganic substances	32.7
Nicotine	67.0
Pyridine	98.0
Ammonia	98.0

#### Other Constituents of Tobacco-Smoke

Laskowski (1951) collected exhaled smoke from smokers, analyzed this and also main-stream smoke, and by difference between these figures determined the amount of a number of cigarette-smoke constituents retained by the lungs. A summary of Laskowski's results is given in Table 1-2.

Biederbeck (1908) found that when 6-8 puffs of ammonia-laden air were taken into the mouth per minute, held there a short time, and then blown into a collection flask, an absorption of 76-88% occurred in the mouth. J. Willke (cited by Biederbeck) had previously found that mixtures of ammonia gas and air sucked into the mouth were absorbed to the extent of 83-90%. About 56% of the ammonia in main-stream cigarette-smoke was absorbed on simple puffing without inhalation, indicating that absorption was much poorer from tobacco-smoke than from air. However, Laskowski (1951) reported that 98.0% of the ammonia in tobacco-smoke was retained in the lungs (if inhalation were practiced).

H. E. Armstrong (1922) stated that, in the absence of inhaling, but little if any carbon monoxide would be absorbed from tobacco-smoke. Baumberger (1923a) reported that in inhaling cigarette-smoke, about 61% of the carbon monoxide was absorbed. Saruta (1937) calculated that the carbon monoxide retained by the body on inhalation of cigarette-smoke amounted to 78% of the total taken into the mouth, and that the amount so retained from a cigarette would saturate 1.7% of the hemoglobin in the circulation with carbon monoxide. Jongbloed (1939) also considered the question of whether or not carbon monoxide could be absorbed through the mucous membranes of the mouth, but did not decide it.

#### FACTORS INFLUENCING THE ABSORPTION OF NICOTINE

##### Animal Experiments

Superficial necrosis of the oral mucosa of cats, produced by scalding or by freezing, retarded absorption or penetration of nicotine (Nacht, 1933). Penetration of nicotine into the skin of rats and guinea pigs was also greatly retarded by prior freezing, scalding, or chemical injury to the skin of the animals (Nacht, 1937); similar results were obtained by damaging the pharyngeal mucosa of cats and rabbits (Nacht, 1937-38, 1935).

Of historical interest in connection with the effect of pH on the penetration of nicotine is the observation by Langley and Dickinson (1889, 1890a) that the superior cervical ganglion required freer application of 1% nicotine sulphate than of 1% nicotine in order to paralyze it. This suggested to the authors that part of the injurious effect of the nicotine directly applied might be due to its alkalinity; but Mitolo (1928), studying this possibility by comparing neutralized nicotine with a solution of sodium carbonate of about the same alkalinity as unneutralized nicotine solution, found that the action of neutralized and unneutralized nicotine was similar, while the sodium carbonate solution had no appreciable effect on conductivity and excitability of toad nerves; therefore, the action of nicotine was not dependent on its alkalinity. Travell (1940a) injected nicotine solutions directly into the lumen of stomachs ligated at the cardia and pylorus in such a manner as to exclude the large blood vessels. In one cat injected with 20 mg./kg. nicotine (15 ml. of solution per kg. body weight) at pH 8.6, the animal died in 41 minutes, and the pH of the recovered stomach contents was 7.8. In a second animal, 50 mg./kg. were injected at pH 1.2: the cat survived, and the stomach contents taken 24 hours later had a

pH of 4.2. From more detailed experiments using other alkaloids, Travell found that the rate of absorption from a stomach varied in general with the pH and with the dose; when gastric juice was rendered alkaline and the alkaloids existed as the free base, absorption was rapid; whereas when the juice was strongly acid and the alkaloids existed as salts, absorption did not occur to any extent. The change in absorption going from alkaline to acid pH depended on the ratio of salt to free base at any given pH; and at any given pH within the range where all the alkaloid was not in the salt form, the size of the dose was a factor in the rate of absorption. Extending her experiments, Travell (1940b) instilled buffered solutions of nicotine into the urinary bladder of cats, the ureters being ligated and the total volume of fluid kept at 5 ml./kg. At a pH of 4.5-6.0, doses of 10 and 20 mg. nicotine were found to have no effect, while similar doses at pH 7.0-7.9 produced fairly marked poisoning; 10 mg. at pH 8.0-9.0 caused severe poisoning, and 20 mg. at pH 9 caused death in two trials. C. H. Richardson (1945) reported that the speed of penetration of nicotine molecules was greater than that of its ions; when tested on rockfishes, appreciable amounts of nicotine were absorbed from solutions in which the alkaloid was almost completely ionized.

Free nicotine applied to the skin of cats was absorbed and resulted in fatal poisoning, whereas the same concentration of nicotine sulphate similarly applied had no effect (Faulkner, 1933). Nicotine base was rapidly absorbed from the skin of the abdomen of rabbits and dogs, whereas nicotine tartrate was only very slightly, if at all absorbed (Manganaro, 1935). Somewhat similar findings were reported to have been by Haag and Neale (1940).

Willenbuecher (1940) studied absorption of 0.11-10.0% nicotine through the shaved skin of dogs. A concentration of 0.11% of the nicotine was absorbed in 60 minutes, compared to 3.5% absorption from a 10% solution. Significant variations between dogs were noted.

#### Absorption in Smoking

Smoking with inhalation greatly increases the absorption of nicotine from tobacco-smoke, compared to puffing (K. B. von Lehmann, 1909; Heinz, 1923; Winterstein and Aronson, 1928, 1929; Bodnar, Nagy and Dickmann, 1935; Pyriki, 1932b, 1943; Wenusch, 1942a, b; Greenberg, Lester and Haggard, 1952; see also Table 1). A few authors, however, have reported a considerable degree of nicotine absorption from puffing in the absence of inhalation (Baumberger, 1923b; Bodnar, Nagy and Dickmann, 1935; Pierce, 1937, 1941); but, even so, less than that observed on inhalation. The more prolonged or the deeper the inhalation, the greater the absorption of nicotine (Winterstein and Aronson, 1928, 1929; Wenusch, 1942a, b; Pyriki, 1943).

As might be expected, the amount of nicotine absorbed is increased in prolonged smoking (W. Straub and Amann, 1940a, b) and by the increasing nicotine content of the cigarettes smoked. According to Pierce (1937, 1941), the average nicotine content of the smoke inhaled during 10 inhalations of the smoke of a cigarette containing 2.27% nicotine was 3.07 mg., while that from a cigarette containing 1.15% nicotine was 1.77 mg. The degree of moisture of the tobacco smoked is also a determining factor, the transfer of nicotine from the tobacco to the smoke being greater with dry than with moist cigarettes and cigars. Retention of the nicotine by the body was greatest when smoking, without inhalation, of the dry cigarettes and cigars, although these differences dis-

appeared on inhalation (Greenberg, Lester and Haggard, 1952). It may be noted in this connection that dry tobacco produces a subjectively more irritating smoke than moist tobacco (Larson, 1952); furthermore, smoke from dry tobacco has a greater edema-producing potency on the rabbit eye (Finnegan et al., 1947).

Certain results obtained by Pyriki (1932b, 1937) suggested to him that, the more the organism was accustomed to smoke, the smaller was the percentage of nicotine retained. On smoking with inhalation, heavy smokers retained 83.92%, moderate smokers 90.10%, and light smokers 95.42% of the nicotine; while smoking without inhalation the nicotine retention in the three groups was, respectively, 4.18%, 4.62%, and 5.00%.

Wenusch has made a particular study of the factors influencing the absorption of nicotine, especially of the differences between cigarette- and cigar-smoking. The more alkaline main-stream smoke of cigars contains part of the nicotine in free form, in contrast to the more acid main-stream smoke of cigarettes, and this free nicotine condensing with the water vapor was said to be absorbed almost quantitatively by the body of the smoker (Wenusch, 1935c). By the nicotine "shift" (proportion of nicotine in the free form), enough nicotine is absorbed, just by mouth smoking and without inhaling, to give the desired physiological effects; cigar-smoke, consequently, does not need to be drawn into the lungs, which would be unpleasant because of its alkaline reaction. Wenusch (1939a) stated that the absorption of nicotine on mouth smoking without inhalation is essentially dependent upon three conditions: the amount of nicotine salt molecules in the smoke drawn in; the length of time the smoke stays in the mouth cavity; and the measure of the agglomerating ability of the nicotine salts. With mouth smoking of the tobaccos of the acid [cigarette] group, only 2-3% of the nicotine in the tobacco smoke is absorbed; with inhalation this can be increased to 10-20%, depending on the depth of inhalation. On deep inhalation, smoke stays in the organism longer, has more time to settle out, and makes many changes of direction, thus increasing the opportunity for agglomerated nicotine salt particles to strike against walls standing at right angles to the direction of flight, and to remain adhered. The procedure by which smoke particles adhere to each other by collision was termed by Wenusch (1940) *zusammenballung* or agglomeration. Agglomerated smoke particles are larger and heavier than single particles, and not only fall to, but also more easily cling to surfaces bordering on the site of the origin of the smoke. The more particles smoke contains in equal volume, the more collisions occur, and the greater the agglomeration. Since cigarette-smoke contains many more particles than cigar-smoke (3 times more condensation particles than cigar-smoke), agglomeration is also much greater. The faster the smoke is moved, and the oftener its direction of motion is changed and the longer motion persists, the greater the agglomeration; and the greater the agglomeration, the more nicotine deposited. On non-inhalation of cigarette-smoke, only about 2% of the nicotine in the tobacco-smoke is retained, because the smoke stays in the mouth only a short time and little movement and change of direction ensue. On inhalation, however, the smoke remains longer in the organism and undergoes more frequent changes of direction, which intensifies agglomeration. Therefore, much more nicotine is retained on inhalation than in puffing. With cigar-smoke, agglomeration does not play a large role, since such smoke contains fewer particles than cigarette-smoke from cut to-

bacco. Cut tobacco (cigarettes) permits some three times as much volatile substances to pass over into the smoke as does uncut tobacco (cigars), because the process of cutting opens up innumerable cells, the contents of which are easily volatilized. The more water vapor (steam) there is in the hot tobacco-smoke, the greater the condensation of the cooled smoke, and the larger the number of condensation particles, the more possibility there is of nicotine absorption. The frequently-made observation that moist cigars are stronger, and moist cigarettes milder, than dry ones was explained by Wenusch (1940) as follows: moist cigars form more alkaline condensate on the surface of the tobacco, and this condensate penetrates deeply into the tobacco and liberates more nicotine, thereby increasing the *Nikotinschub* or "nicotine shove." Moist cigarettes seem milder than dry ones because more air must be drawn through moist tobacco to burn it than through dry tobacco; the minute particles in the smoke of moist tobacco are distributed through a larger volume than in smoke from dry tobacco, whereby agglomeration of the nicotine salts is less. With cigar-smokers, then, the amount of nicotine deposited in the organism depends on the size of the "nicotine shove." In a long cigar, the "nicotine shove" precipitates for the most part in the unsmoked remainder, and the shorter the butt, the greater the "nicotine shove" deposited in the mouth by each puff, in contrast to the situation with cigarette-smokers, wherein each puff, from first to last, deposits almost equal amounts of nicotine in the organism, and the butt length plays no role. To sum up, the nicotine uptake by the organism depends not on the nicotine content of the smoked tobacco, but rather on such factors as the acidity or alkalinity of the tobacco smoke, the agglomeration of nicotine salts or particles, and on *Nikotinschub*.

Pyriki (1943) stated that tobacco chemistry (the work of Wenusch described above) had introduced the concept of agglomeration of nicotine salt particles through the resinous constituents of the smoke, leading to the idea that the human organism retained more nicotine from the main-stream smoke of tobacco rich in resins [cigars] than from tobacco poor in resins [cigarettes]. However, through a series of experiments on man, Pyriki felt that he had clearly established that physiologically this conception did not hold.

W. Straub and Amann (1940a, b) considered that, in nicotine absorption in smoking, the absolute quantities of nicotine were less decisive than the temporal conditions of the absorption. Among the factors involved in the latter are the pH of the body fluids (the higher the pH, the faster are the undissociated nicotine molecules absorbed) and the steepness of the so-called "nicotine current." According to Wenusch (1940), the reason why a cigar smoked in a holder becomes milder, while a cigarette smoked in a long holder becomes stronger, is that in the former instance free nicotine is deposited in part in the holder and therefore does not reach the organism, whereas in the latter case, lengthening the passage through which the cigarette-smoke passes increases the possibility of agglomeration.

To sum up, the rate and amount of absorption of nicotine by the smoker depend to a greater or lesser extent upon the following factors:

1. length of time the smoke remains in contact with the mucous membranes;
2. pH of the body fluids with which the smoke comes in contact;
3. degree and depth of inhalation;
4. degree of habituation of the smoker (?);

5. nicotine content of the tobacco smoked;
6. moisture content of the tobacco smoked;
7. form in which tobacco is smoked (cut [cigarettes] or uncut [cigars]) (?);
8. length of butt;
9. use of holder or filter;
10. alkalinity or acidity of the tobacco smoke (?);
11. agglomeration of smoke particles (more important in cigarette-smoking).

#### *Non-Pulmonary Absorption of Nicotine in Man*

Wolters (1898) reported that a preparation of nicotine salicylate made into a 0.1% ointment with lanolin did not cause any symptoms of poisoning upon inunction, whereas treatment with nicotine soap caused marked intoxication symptoms in 2 of 8 patients.

Moyer and Maddock (1940) suggested that swallowed nicotine (as nicotine or as nicotine dissolved in saliva and swallowed by non-inhalers) produced little effect because intestinal absorption permitted detoxication by the liver.

#### *Membrane Permeability*

##### *Artificial Membranes*

Weatherby (1942b, 1943a) found that if lecithin and cephalin, or either alone, were dissolved in collodion solutions, and membranes prepared from this mixture, such membranes often exhibited asymmetry potentials of considerable magnitudes, the potentials varying with changes in pH. Apparently, lecithin contributed the positive component of the potential, which was most evident at relatively high hydrogen-ion concentrations, and cephalin contributed the negative component, evident at a hydrogen-ion concentration of approximately pH 5 and above. Alkaloids such as nicotine permeated these membranes readily from an alkaline medium, but not from an acid medium in which the nicotine would be positively charged by virtue of existing as the acid salt. Tested on a living system, it was found that the toxicity to parameria of nicotine solutions at various pH values approximately paralleled the permeability of artificial membranes containing lecithin and cephalin at these same pH values. Thus, such artificial membranes exhibited some of the properties of semipermeability generally associated with membranes of living cells. The explanation proposed for these phenomena was based on ionization of the lecithin or cephalin molecules contained in the membrane, the nature of the ionization depending on the pH of the medium in contact with the membrane. Ions bearing charges of similar sign to that of the membrane are repelled by the membrane, and in this manner penetration of the membrane is prevented. Un-ionized molecules are not repelled in this manner, however, and penetration may occur more or less readily. A definite correlation was found to exist between rate of penetration and concentration of un-ionized molecules of acid or base. Weatherby (1943b) then tested the validity of this hypothesis by making relatively simple changes in the molecule so as to alter its ionization, and hence supposedly its permeating characteristics. Derivatives of nicotine in which methyl iodide was added to one or the other, or both, of the nitrogen atoms contained in the molecule were tested. Such compounds would be expected to ionize under all conditions of pH, and consequently should not penetrate membranes containing lecithin and cephalin; and this was found to be the case. Nicotine, which at pH 7.4 exists as the free base to the extent of only 2%, compared to 16% for nicotine

at the same pH, permeated the artificial membrane much more slowly than nicotine under similar conditions. However, when the pH of the norm nicotine solution was raised to 9.5, the rate of permeation was greatly increased, and compared favorably with the rate of permeation of nicotine from a solution containing the same proportion of free base. These observations lent added theoretical support to the findings of Larson and Haag (1943), who reported that nicotine was more toxic to mice on intraperitoneal injection than was norm nicotine, due to the greater availability of the free base of nicotine compared to norm nicotine, the higher pH of norm nicotine compared to that of nicotine resulting in a greater ratio of salt to base at body pH in the case of norm nicotine (see Chapter 14, relationship of chemical structure and toxicity).

Fleckenstein, Gunther and Winker (1951) found that when 0.02 M nicotine hydrochloride was added to 0.01 M potassium chloride on one side of a collodion membrane, with 0.001 M potassium chloride on the other side, a fairly marked reversal of membrane potential occurred. Adsorptive binding of nicotine took place on the membrane, but this was reversible on repeated washing. Corresponding to their antagonistic effect against nicotine (see also Chapter 14, page 457, the influence of proteins on nicotine toxicity), the local anesthetics exhibited almost irreversible binding.

#### Tissues

Langley (1909) found that nicotine was adsorbed by all tissues; the amount taken up by frog muscle increased with the percentage of nicotine in the fluid in which the muscle was immersed. Burridge (1911) reported that frog gastrocnemius muscles placed in 0.5–1% nicotine in Ringer's solution for 24 hours gained in weight, but that the weight gain was independent of nicotine concentration. According to Langley (1909), curare did not prevent the adsorption of nicotine by frog muscle, but M. Lapicque (1921), who found that frog gastrocnemius, dipped in hypotonic salt solution containing 5% nicotine, swelled more than muscle dipped into the same fluid without nicotine (the increase in imbibition being correlated with the decrease in chronaxie observed during the first phases of nicotine poisoning), reported that curare (which increased the chronaxie of muscle) decreased its imbibition. Isolated frog gastrocnemii contracted by 1:2000 nicotine imbibed a smaller amount of water, when immersed in distilled water, than did normal muscle (Gottlieb, 1934, 1935).

Experiments *in vitro* and *in vivo* have shown that red blood cells take up nicotine (Scharpfi, 1921; Burstein, 1932; Guidetti, 1937; Tsujimoto *et al.*, 1955); the plasma to erythrocyte ratio of nicotine distribution increased with the dose or concentration of nicotine (Burstein, 1932).

In animals, the placenta was found to be permeable to nicotine (Holstetter, 1923; Morra, 1935; Romaniello, 1939; Sergueev, 1939, among others), and placental permeability to nicotine has been deduced in pregnant women from the fact that maternal smoking of a test cigarette caused increases in fetal heart beat (Sontag and Wallace, 1935; among others). Nicotine did not alter the permeability of the placenta sufficiently to permit passage of Trypan blue or Congo red, nor was placental permeability to sodium iodide or sodium bromide altered by small doses of nicotine, although large doses increased placental permeability to sodium bromide (Sergueev, 1939).

Bellarmino (1893) tested the effect of nicotine on the absorption of fluorescein into the aqueous humor by applying 2% nicotine to the right eye of rabbits, then washing out and

maintaining fluorescein solution in both eyes for 20 minutes. In this way, it was demonstrated that nicotine decreased the diffusion of fluorescein into the aqueous humor.

According to Zettler (1937), 1:1000–1:50 nicotine, when effective, decrease the permeability of isolated arterial segments, the permeability being measured by diffusion of a suitable material from the perfusion fluid through the vessel into the isolated organ-bath.

Osterhout (1919) found that salts which had opposite effects on permeability were able to antagonize each other; thus, sodium chloride produced an increase in permeability, while calcium chloride produced a decrease. To test the antagonism between salts and alkaloids, nicotine in varying amounts was added to 0.52 M sodium chloride, and its effect upon the electrical conductivity of *Laminaria* (kelp) was determined. Nicotine antagonized the action of sodium chloride by inhibiting the fall of resistance which occurred in pure sodium chloride.

Beutner (1925) tested the binding power of serum from different species for various alkaloids by dialyzing the serum-alkaloid solution against pure water using parchment paper shells. According to Storm van Leeuwen (1924), nicotine was tried with this technique with negative results. However, since the reagent used for the detection of nicotine was mercury potassium iodide, it may be that this simply was not sensitive enough to detect small amounts of adsorption.

Walter (1944) stated that the penetrability of drugs through the oral mucosa was favored by a high fat-water distribution coefficient or by an exceptionally high fat solubility, and he pointed out that nicotine had a unique solubility in fats, being completely miscible in all proportions, and that this accounted for its absorbability, since its fat-water distribution coefficient is low (2.6). Okumura (1937b, 1938) attempted to correlate the effects of nicotine with its tissue-blood partition coefficient; this varied in different tissues, different species, and in the presence of habituation.

#### Membrane Potentials

The average membrane potential of the relaxed rectus abdominis muscle of the frog was found to be 30–34 millivolts; after exposure of the muscle to 1:5000 nicotine for 3 minutes, the average potential dropped to 16.6 millivolts (Fleckenstein, 1950; Fleckenstein, Wagner and Gögge, 1950). In frog sartorius nerve-muscle preparations at room temperature, nicotine caused a brief depolarization of the end-plate regions, which subsided spontaneously without removal of the nicotine, with restoration of the membrane potential to about its normal value (Thesleff, 1955). A detailed discussion of potential changes in muscle is given in Chapter 4.

#### Phase-Boundary Potentials

Barnes and Beutner (1949) pointed out that adrenergic drugs dissolve in triglyceride oils, on which they form negative phase-boundary potentials; in contrast, the cholinergic drugs are inactive in adrenergic oils. Nicotine was found to have the solubility characteristics of adrenergic drugs, giving 87 millivolts on tributyrin, suggesting that the pressor effect is a direct adrenergic action. Using oil cells similar to those described by Beutner and Barnes, (Science 110: 511, 1941; see also Barnes and Beutner, J. Cellul. Physiol. 20: 307, 1942), with re-distilled guaiacol for the oil phase, Exley and Hey (1955) found that the stimulant activity of nicotine acid tartrate was not associated with a high boundary potential.

## FATE

## DISTRIBUTION

## Blood

Following a 120-minute exposure of the legs and parts of the wings of cockroaches and various locusts to nicotine vapor, nicotine could be detected in the blood and organs of the insects (C. H. Richardson, Glover and Ellisor, 1934).

Noether (1923) found small amounts of nicotine in the blood of guinea pigs 6 hours after subcutaneous injection of approximately 10 mg./kg. Following intracardial injection of 20 mg. of nicotine in guinea pigs, Werle and Meyer (1950) found 0.038 and 0.066 mg./gm. in the blood. In 2 animals exposed to cigarette smoke until death, blood concentrations of nicotine found were 0.029 and 0.046 mg./gm.

A. S. Taylor (1855a, b, c) demonstrated the presence of nicotine in the blood, but not in the liver and lungs, of a rabbit killed by a drop of nicotine placed in the mouth. Following intraperitoneal injection of 15 mg. nicotine into rabbits, blood drawn during or after convulsions gave negative tests for nicotine using a turbidometric method with Lugol's solution, but yielded positive results for nicotine when the steam distillate was bioassayed on frog gastrocnemius; control blood from non-nicotinized rabbits gave no reaction (Sokolov and Lyubovtzeva, 1923). Thirty minutes following subcutaneous injection of 9-15 mg./kg. nicotine into rabbits, the blood concentration of nicotine, as determined by leech-muscle bioassay, was equivalent to a 1:40,000-1:35,000 solution (Sergueev, 1939). Following hypodermic injection of 5 mg./kg. nicotine to rabbits, the peak plasma nicotine concentration of 4 gamma/ml. was found at 30 minutes, with a gradual fall over the next several hours; after 5 hours nicotine could not be demonstrated in the blood plasma (Tsujimoto et al., 1955).

Assuming that a cat's blood amounts to 7% of its body weight, W. Stäub and Amann (1940) calculated that the nicotine concentration in the blood, at least momentarily after intravenous injection of the M.L.D. of 1.5 mg./kg. nicotine, would be about 1:50,000. As noted above, according to Sergueev (1939), the blood concentration of nicotine 30 minutes after subcutaneous injection of 9-15 mg./kg. nicotine into rats was equivalent to a 1:40,000-1:35,000 solution.

Orfila (1851b, c) found nicotine in the blood of dogs poisoned with this substance. Tsujimoto and coworkers (1955) reported that following hypodermic injection of 5 mg./kg. nicotine to dogs, peak plasma values of 1.7 gamma/ml. appeared in 15-30 minutes, and fell to 1 gamma/ml. in 6 hours; nicotine was determined by the CNBr-aniline reaction. Peak signs of poisoning corresponded to peak blood values.

Following fatal nicotine or tobacco poisoning in man, nicotine has been found in the blood (Thélin and Wehrli, 1935) or in a mixture of viscera and blood (A. Esser and Kühn, 1935; Detis, 1937).

In rabbits and dogs given subcutaneous injection of 5 mg./kg. nicotine, the blood plasma to erythrocyte ratio of nicotine distribution was found to be 10:7 (Tsujimoto et al., 1955). In dogs, upon the intravenous or subcutaneous injection of 0.3 mg./kg. nicotine, nicotine was present in the cell fraction, but absent from the plasma; with 0.5 mg./kg., the nicotine was divided about equally between the two fractions; and after 1 mg./kg., nicotine predominated in the plasma (Burstein, 1932). In experiments on fresh calf's blood *in vivo*, Schaeppi (1921) showed that nicotine distributed itself proportionately between the plasma and the blood cells. In similar experiments on dog blood, Burstein (1932) found that

when the nicotine did not exceed 0.0075 mg. per gm. of cells, it was adsorbed, and none appeared in the plasma (this figure corresponded exactly with the *in vivo* results), while nicotine in excess of this quantity appeared in the plasma.

Barry (1925) estimated that 1-2 mg. of nicotine per liter of blood was not an excessive estimate in the case of a heavy smoker. Guidetti (1937), measuring nicotine by the degree of opalescence produced when phosphotungstic acid was added to aqueous solutions containing the alkaloid, found that blood from 2 non-smokers showed no opalescence, while the test was positive in varying degree with the blood of smokers. When a non-smoker smoked 1 cigarette every half hour for 6 such periods, the first 3 samples showed no opalescence, but the succeeding 3 showed increasing opalescence. When the plasma was separated from the cells, the test for nicotine on plasma alone was negative, while the cellular fraction was positive. Guidetti thus agreed with Burstein (1932) that the nicotine was held by the corpuscular fraction. According to W. A. Wolff, Hawkins and Giles (1948), the blood of smokers who had smoked 20 or more cigarettes during the day contained 0.10-0.28 gamma nicotine per ml. These same authors (1949) reported that smokers who had abstained from smoking for 8-10 hours showed the presence in their blood of nicotine or some nicotine-like substance in the amount of 0.02-0.35 mg. per liter. In 15 subjects who smoked 20 cigarettes of standard brand in 7 hours, the increase in blood nicotine ranged from 0 to 0.13 mg. per liter; the increases among the deep inhalers appeared to be greater than among the slight inhalers. Experiments on pipe- and cigar-smoking were made on cigarette-smokers who switched for this test and tried to smoke an amount equivalent to 20 cigarettes; the increases in blood nicotine found appeared to be of the same order as in cigarette-smoking. In the above experiments, nicotine was analyzed by the method of Wolff and Hawkins (1948), and the material was also bio-assayed on blood pressure (cat); the pressor response in the latter case was identical to that obtained from the same quantity of nicotine.

Using the chemical method of Wolff and Hawkins (1948), Wolff and Giles (1950) determined blood-nicotine levels in habitual tobacco-chewers. In 21 normal adult males who chewed 15.6-35.4 gm. of tobacco over 6.5-8 hours, the increase in blood-nicotine concentration during the chewing period averaged 0.03 mg. per liter, a value slightly lower than that obtained with comparable doses of nicotine from smoking.

## Tissues

C. H. Richardson, Glover and Ellisor (1934) detected nicotine in the organs as well as the blood of cockroaches and locusts exposed to nicotine vapor. Following exposure of imagoes and older juvenile forms of the cockroach (*Periplaneta americana*) and larvae of the nocturnal moth (*Heliothis obsoleta* Fabr.) to nicotine vapor, Glover (1936) was able to detect nicotine in the body and its various organs. In the cockroach, nicotine was found chiefly in the cuticula, the cells of the alimentary canal, and in the nerve tissue.

Ellisor and Richardson (1935) immersed goldfish (*Carassius auratus*) under controlled conditions in nicotine solutions of 0.002 M, 0.001 M, and 0.0002 M concentrations, and found that the mean body concentrations of nicotine at death approached 0.034 mg. of nicotine per gm. of tissue; the higher the pH of the nicotine solution, the greater the mean body concentration. Tissue analyses showed that the greatest concentrations were always found in the dermal tissue, and lesser amounts were present in muscles and inner tissues. Nicotine

TABLE 1-3  
Distribution of Nicotine in the Organisms

Species	No. in Series	Hrs. after Nicotine Inj. or Smoke Administration	Nicotine or Smoke Administered	Nicotine, gamma/gm. or ml.											
				Liver	Lungs	Kidney	Brain	Muscle	Thymus	Heart	Spleen	Pancreas	Greater omentum	Blood	Plasma
Mouse*	6	3	Nicotine, 2 mg./kg., i.v.	.048	.032	.052	.009	.002		.024	.020				
	6	6		.054	.019	.024	.005	.002		.022	.036				
Guinea pig†	2	[? to death]	Nicotine, 20 mg., i.c.	108	04	49	214	58						.63	
	2	to death	Cigar-smoke exposure	101	20	30	23	17	13					.38	.12
Dog*	1	1	Nicotine, 8 mg./kg., s.c.	8.6	6.1	14.3	13.7	7.0	10.8	7.8	8.0	13.9	8.6		4.2
	1	3		6.1	8.0	9.6	6.9	6.4	6.6	6.1	5.6	7.2	6.7		2.0
	1	6		3.4	2.9	3.7	2.5	4.1	2.9	3.4	3.0	4.1	2.9		1.0
Rabbit*	1	1	Nicotine, 5 mg./kg., s.c.	3.3	4.2	8.9	3.7	4.8	6.8	5.2	4.0	7.9	3.6		3.0
	1	3		1.7	0.8	2.5	0.8	1.1	1.3	0.8	1.0	1.0	0.8		1.0
	1	6		0	0	0	0	0	0	0	0	0	0		0
Rat†	1	24	Nicotine, 5 mg./kg., s.c.	0	0	0	0	0	0	0	0	0	0		0

\* Ganz, Kelsey and Geiling (1951): nicotine randomly labeled with C<sup>14</sup> (activity equal to 501,000 cpm/mg.; nicotine concentration = C<sup>14</sup> content calculated as picoliter.

† Werle and Meyer (1950): nicotine determined by method of Werle and Becker (1942).

\* Tsujimoto et al. (1958): nicotine content determined by the CNBr-aniline reaction; controls run on tissues of untreated animals, to which nicotine was added.

† Werle and Uschold (1945): tissues steam-distilled in the presence of sodium chloride and magnesium oxide.

determinations were made by the method of Glover and Richardson (Iowa State Coll. J. Sci. 10: 249, 1936).

Orfila (1857b, c) stated that he had detected nicotine in the liver, kidney, lungs, and the blood of animals poisoned with nicotine. Graziani (1893) isolated crystals of nicotine di-iodide from the tissues of 2 rabbits killed by injection of nicotine or infusion of tobacco; from the tissues of 2 guinea pigs similarly treated, nicotine could not be isolated. Langley (1909) stated that nicotine was absorbed by all tissues, the amount taken up being proportional to the nicotine concentration.

Morin (1952a, b) examined the liver and lungs of a determined snuff-taker who died aged 70; the residue, after standard alkaloidal extraction procedures, presented the odor of nicotine, and reacted like nicotine with various reagents.

Quantitative estimation of nicotine concentrations in various tissues following injection of nicotine or exposure to tobacco-smoke have been made by a number of workers (Table 1-3). Their results, however, are not comparable, since the experimental conditions varied widely; moreover, the different methods of analysis used by the several investigators also tend to invalidate any comparison of results.

Noether (1923) examined the tissues of guinea pigs 6 hours after subcutaneous injection of 10 mg./kg. nicotine. Estimated by bio-assay on leech muscle, nicotine was found in the urine in greatest concentration; small intestine, liver, and lung followed in that order. The mucous membrane of the larynx contained no demonstrable amount (this tissue was examined because of the irritating and burning sensation that occurs there after subcutaneous injections of nicotine). Small

amounts of nicotine, as noted above, were present in the blood.

Fabre and Perdreau (1942) exposed a white mouse to the smoke of 7 cigarettes in 2 hours; between cigarettes, the animal was taken out of the smoke chamber to prevent asphyxiation. At the end of the experiment, its viscera were found to contain 0.2 mg. nicotine. Another mouse was similarly exposed to the smoke of 10 cigarettes; its viscera contained 0.4 mg. nicotine. Nicotine was determined following steam distillation by the method used by Corcoran et al. (1939).

Werle and Uschold (1945) could find no nicotine in the tissues of rats 24 hours after injection of 5 mg./kg. nicotine; the tissues were steam-distilled in the presence of sodium chloride and magnesium oxide (Table 1-3).

Werle and Meyer (1950) injected guinea pigs with approximately 50 mg./kg. nicotine intracardially. The organs were removed immediately after death of the animal, placed on ice, and then ground with sand, distilled, and the nicotine determined by the method of Werle and Becker (1942). Results of the analyses are given in Table 1-3; the relatively high brain concentration of nicotine was considered to be worthy of note. Werle and Meyer also exposed guinea pigs to cigar-smoke in a desiccator until death, following which the tissues of 2 of the animals were immediately analyzed for nicotine (Table 1-3), while in the case of 2 other animals, the organs were kept in a refrigerator for 24 hours and 72 hours, respectively, before analysis. In comparison with analyses made immediately after removal of the organs, analysis of organs kept in the refrigerator for 24 hours before analysis gave results which were 50-75% lower than those analyzed immedi-



ately, while after 72 hours in the refrigerator, the nicotine content of the tissues (except in brain) was extremely low. These latter findings point up the importance of prompt tissue analysis for nicotine in distribution studies. The results of these studies show that the nicotine distribution does not parallel the vascularity of the organ. Following intraperitoneal injection, the nicotine distribution in the organs was said to correspond to the expected, although the nicotine concentration in muscle was considered to be surprisingly low.

Gans (1919) reported making distribution studies in mice, rats, and guinea pigs following intravenous injection of radioactive nicotine. Following intravenous injection of nicotine randomly labeled with  $C^{14}$  into mice, Gans, Kelsey and Geiling (1931) found that the livers showed the highest radioactivity of the specific organs studied, skeletal muscle and brain the lowest, while lungs, spleen, heart, and kidney showed intermediate values (Table 1-3). When the isolated heart of the guinea pig was perfused with approximately 100 ml. of a 0.1 mg./ml. solution of radioactive nicotine (randomly labeled with  $C^{14}$  activity equal to 501,000 cpm/mg.), an appreciable uptake of the drug occurred only during the initial period of heart blood with a perfusion rate of about 4 ml. per minute, the nicotine uptake during this period amounted to about 320 gamma (Gans, Kelsey and Geiling, 1931; Geiling, 1931). Following this a dynamic equilibrium may have been established, in which release of nicotine from the heart was equal to the uptake. That such a release could occur was shown by the rapid washout of almost all of the radioactivity taken up by the heart during the stoppage period when the radioactive perfusion was followed by perfusion with normal Ringer-Locke solution. As the conclusion of the perfusion with radioactive nicotine the heart tissue was subjected to analysis, and no differential distribution of radioactivity in the auricles, ventricles, and septum was found.

Tajimoto and co-workers (1933) found the tissue distribution of nicotine in dogs 1 hour after subcutaneous injection of 5 mg./kg. nicotine to be higher in lungs, pancreas, and brain than in any other tissue. In the rabbit, the results were almost the same, except in brain. Six hours after injection, nicotine could not be demonstrated in any tissue by the CNBr-aniline method of estimation (Table 1-3). Controls were run on tissues of untreated animals to which nicotine was added.

According to Busse and Lendle (1931), calculations on nicotine absorption during smoking indicated that body concentrations of 1:30 million-1:3 million were obtained.

#### After Chronic Tobacco Administration

Kobos (1897) administered, by way of the food, 0.2-0.25 gm. tobacco 3 or 4 times daily for over 2 months to 3 dogs, 3 rabbits, and 3 guinea pigs. In all cases, the greatest portion of the nicotine was found in the cardiac muscle, the liver, spleen, and intestinal tract, although the animals when sacrificed had not received tobacco for at least 6 days. The muscle juice and that of the liver of animals slowly intoxicated by tobacco was toxic; intravenous injection of 2-3 centigrams producing serious convulsions in a rabbit weighing 1.15 kg.

Okumura (1937b, 1938), found, in rabbits, that the blood-tissue partition coefficient was altered by repeated subcutaneous injection of nicotine; gradually, more nicotine was absorbed by the blood and less by the different organ tissues (liver, muscle, etc.), especially by central nervous tissue (cerebrum, spinal cord). In contrast to the rabbit, in dogs repeatedly injected with nicotine, not only the adsorption capacity of the blood, but also that of organ tissues, and es-

pecially that of central nervous tissue, was increased, so that the tissue-blood partition coefficient was not decreased, but increased. Okumura used these findings to explain why rabbits became habituated to nicotine, while the dog did not, but instead by repeated poisoning became more sensitive to the drug [but see Chapter 15 for evidence that tolerance does develop in the dog].

#### In Fatal Tobacco or Nicotine Poisoning in Man

Post-mortem analyses of tissues for nicotine content in fatal acute tobacco or nicotine poisoning in man have been reported by Rabot (1886) [liver]; A. Esser and Kühn (1933) [pooled stomach, esophagus, liver, kidney, blood]; Kratz (1935) [pooled brain, liver, heart, kidney, spleen]; Palmer (1935) [pooled liver, spleen, kidneys]; Orsós (1936) [brain]; Detis (1937) [all the viscera, pooled; blood]; Thelin and Wehrli (1938) [blood]; Moller and Simesen (1939) [spleen, liver, blood, kidney, brain]; and G. S. Smith (1951) [stomach, intestine, liver, kidneys-spleen-heart, brain]. Details of these cases may be found in Chapter 14.

In connection with post-mortem analyses for nicotine, the experimental work of Melsens (1857-58) should be mentioned at this point. Melsens studied the detection of nicotine in the organs (tongue, stomach, lungs, liver, etc.) of nicotine-poisoned animals, usually dogs; the organs were allowed to putrefy for periods up to 7 years prior to attempting to detect the alkaloid. The author concluded that one could detect nicotine long after death, and that the phenomena of slow putrefaction at low temperature in the absence of air did not destroy nicotine, while negative results could be expected at elevated temperatures and in the presence of air under circumstances where nicotine base could be volatilized or altered. However, it will be recalled that Werle and Meyer (1950) found that liver, lung, kidney, and muscle nicotine concentrations decreased progressively with the time elapsing between death of the poisoned animal and analysis of its tissues, even though the organs were kept in the refrigerator. In the case of liver, the average nicotine concentration was 100 gamma per gm. of tissue on immediate analysis, and only 2.5 gamma after 72 hours.

#### EXCRETION

##### Urine: Animals

Langley and Dickinson (1890a) stated that nicotine was readily found in the urine of rabbits, cats, and dogs, following injection of nicotine. Noether (1923) found nicotine in the urine of guinea pigs within 1.5 hours after subcutaneous injection of the drug. Excretion reached a peak at 5-7 hours, and fell away to trace quantities at 10 hours. In 8 dogs given 2-3 mg. nicotine intravenously, Corcoran, Helmer and Page (1939) found that recovery from the urine varied from a maximum of 12.8% in 2 hours to a minimum of 4.5% in 24 hours; and in dogs given 3 mg./kg. nicotine subcutaneously in divided doses, Larson and Haag (1942a, b) found about 10% was excreted unchanged.

Using dogs under Dial anesthesia and artificial respiration during periods of respiratory paralysis, Finnegan, Larson and Haag (1947a) showed that the per cent of administered nicotine excreted unchanged in the urine increased with increasing nicotine dosage. Thus, whereas the percentage excreted in the urine averaged only 6.7 following 3 mg./kg. intravenous doses, following 15, 24, and 48 mg./kg. amounts, it rose to 13.7, 20.0, and 30.4, respectively; this is in line with the further finding that with increasing nicotine concentration, the rate



of urinary excretion of nicotine increases linearly, whereas rate of detoxication within the body increased logarithmically. Urinary excretion of nicotine was virtually complete within 16 hours after cessation of administration.

In a comparable study on norm nicotine, Hucker and Larson (1938) found that the per cent of administered norm nicotine excreted unchanged increased much more rapidly with increasing dose than was true for nicotine, and reached a peak of about 60% at dose levels of 15 mg./kg. and above. In this case too, urinary excretion was virtually complete within 16 hours following cessation of administration.

Yamamoto, Takeuchi and Tsujimoto (1934) gave 12-15 mg. nicotine subcutaneously to 17 male rabbits, and collected the urine, which was steam-distilled before analysis. An average of 7.7% of the injected nicotine was excreted in 24 hours; 60% of this appeared in the first 6 hours. Only traces appeared in the second 24 hours.

Ganz and Kelsey (1950) injected 5-10 mg./kg. of radioactive nicotine (randomly labeled with  $C^{14}$ ; activity equal to 501,000 cpm/mg.) into rats, and determined urinary nicotine output by both radioactive tracer and spectrophotometric techniques. They found an 8-12% excretion of unchanged nicotine, with good agreement between the two analytical techniques. Following subcutaneous injection of 1.5-2.5 mg./kg. radioactive nicotine in rats, the urinary excretion of radioactivity (nicotine plus its metabolites) began almost immediately after injection, and was practically complete after 16 hours. About 40% of the injected activity was excreted in 3 hours, about 65% in 6 hours, and all or almost all, in the urine after 16 hours; about 25% constituted unchanged nicotine (Ganz, Kelsey and Geiling, 1951; Geiling, 1951).

Werle and Meyer (1950) injected 6 rats intraperitoneally with 5-10 mg./kg. nicotine, and collected the urine at 1-2 hours intervals for nicotine. Results were given graphically in terms of maximum extinction plotted against hours after injection of nicotine; the curve rose to a maximum at 3 hours. In dogs given 5 mg./kg. nicotine by slow intravenous infusion, about 10% of the nicotine was excreted unchanged.

In chronic experiments, Kohos (1927) found the urine of dogs, rabbits, and cats fed 0.2-0.25 gm. tobacco 3 or 4 times a day for over 2 months to be non-toxic or intravenous injection in a rabbit, which was taken to be an indication that elimination of nicotine was very slow. Evidence has also been presented for the dog and the rabbit (Okamoto, 1929) and for the rat (Werle and Uschold, 1945) that chronic exposure to nicotine leads to a decreased percentage excreted unchanged in the urine. Werle and Uschold (1945) injected 4 rats with 5 mg./kg. nicotine daily for 10 days, and reported the average per cent nicotine excretion in the urine as 20.9, 17.5, 14.7, 12.3, 10.2, 8.9, 7.3, 6.4, 5.9, and 5.9 on successive days. Since chronic nicotine injection had been found to increase the detoxifying power of the tissues, the authors concluded that, with increased detoxification power for nicotine, less was excreted in the urine. With both non-habituated and habituated rats, urinary excretion of nicotine required 9-10 hours.

So far as the mechanism of urinary excretion of nicotine is concerned, the only published data appear to be those of S. Ozawa (1929, 1930). According to this author, nicotine was eliminated in the load from both glomeruli and tubules. Investigation of the site of nicotine excretion in the mammalian kidney, and factors affecting this, is greatly to be desired.

#### Urine: Man

Noether (1923) demonstrated nicotine by leech-muscle bio-assay in the urine following the smoking of 1 cigar or 2 cig-

arettes. After a 12-hour abstinence, the urine of heavy smokers was free of nicotine, but 2 hours after the morning cigarette, nicotine was again present in the urine. On smoking a cigar, nicotine was present in the urine in 1.5 hours, reached a peak at 2.5 hours, then fell off, and none could be demonstrated at 12 hours.

Heiduschka and Muth (1927), by means of a bio-assay on ciliata, detected nicotine in the urine after the smoking of so-called nicotine-harmless cigarettes.

Using the procedure of Noether, Emanuel (1931) found nicotine in the urine of each of 10 women who smoked 6-15 cigarettes in 1-2 hours; the amount in any one test-period (2-3, 4-5, and 7-8 hours after smoking) varied from 0 to 0.03 mg. per liter.

Fretwurst and Hertz (1932) were not able to isolate nicotine (as the dipicrate) from the urine of moderate smokers.

Bodnar, Nagy and Dickmann (1935) recovered 1.31 mg. nicotine in the urine following smoking of 10 cigarettes with inhalation; after smoking the same number of cigarettes without inhalation, no nicotine was found in the urine. The method used for the detection of urine nicotine was that of Nagy and Dickmann (Zschr. anal. Chem. 94: 12, 1933).

Helmer, Kohlstaedt and Page (1939) isolated nicotine as the oxalate and picrate from the urine of smokers, and completed the identification through melting-point and empirical formula determinations and pharmacological tests. Most of the nicotine disappeared from the urine within 3-4 days after smoking had been discontinued. Corcoran, Helmer and Page (1939) reported that the amounts of nicotine excreted in the urine of persons who smoked varied from 1.4-9.8 mg. per 24 hours. Urinary excretion of nicotine tended to increase with the number of cigarettes smoked, and averaged 0.234 mg. (range, 0.06-0.49 mg.) per cigarette. By the analytical method used (and described in this paper), the urine of non-smokers gave an average color equivalent to 0.233 mg. of nicotine (range 0.06-0.42 mg.); the authors suspected that a part of this blank was due to nicotinic acid.

According to Fabre and Perdreau (1942), a human subject remaining in an experimental chamber filled with cigarette-smoke for 3 hours excreted 0.2 mg. nicotine in 200 ml. of urine produced during this period. Another subject who stayed for 3 hours in a play-house which was particularly smoky excreted 1.2 mg. nicotine in 24 hours. Nicotine was determined by the method of Corcoran, Helmer and Page (1939).

Perlman, Dannenberg and Sokoloff (1942) studied urinary excretion of nicotine in 55 white female habitual smokers aged 16-36; pooled morning (7 a.m.-1 p.m.) and afternoon (1 p.m.-7 p.m.) urine samples were bio-assayed for nicotine by the *Daphnia magna* method. In occasional smokers (1-4 cigarettes daily), nicotine content in the morning urine averaged 1.657 mg. per liter, in afternoon urine, 2.75 mg. per liter. In moderate smokers (5-10 cigarettes daily), the morning urine contained on the average 2.625 mg. nicotine per liter, the afternoon urine, 3.645 mg. per liter. In heavy smokers (11-20 cigarettes daily), nicotine content of the morning urine averaged 4.9 mg. per liter, that of afternoon urine, 6.62 mg. per liter. There was thus a definite correlation between the number of cigarettes smoked and the quantity of nicotine excreted in the urine.

Bodnar and Novak (1934) reported results of analyses for 24-hour urinary excretion of nicotine by 8 smokers. Under an erroneous assumption that 40% of the nicotine content of the tobacco was absorbed, urinary excretions of 0-3.9% were calculated.

Using semi-quantitative paper chromatography, Lickint

and Lukesch (1956) determined that maximal urinary excretion of nicotine occurred 3.5-4.5 hours after beginning of smoking 5-10 cigarettes. After smoking 1 cigarette, elimination of nicotine was complete in 15 hours, but after 10 cigarettes smoked within 2 hours, traces of nicotine were still detected with certainty in the urine after 48 hours. Also using paper-chromatographic technique, Jatzkevitz (1953) detected nicotine in the urine 18 hours after the smoking with inhalation of 1 cigarette. Quantitative estimations on the urine of tobacco-users show that the amount of absorbed nicotine excreted unchanged is of the order of 10% or less (Corcoran, Helmer and Page, 1939; Haug and Larson, 1942; Wolf and Giles, 1950). Haug and Larson (1942) found that this amount varied somewhat with the pH of the urine, less being excreted unchanged when the urine is maintained alkaline (about 2-4% at pH 7-7.5) than when it is maintained distinctly acid (10-13% at pH 5-5.5). The explanation offered by these authors was based on the pK<sub>a</sub> of nicotine, which is such that large changes in the ratio of free to combined base are produced by variations in pH within the range compatible with living tissues. The free base is much more readily absorbed from the urinary tract than are salts of nicotine, and hence at the higher urinary pH's, more of it may become re-exposed to detoxication within the body. Incidentally, the data of Haug and Larson indicate little possibility of reabsorption of nicotine from the bladder of smokers, to the point of poisoning, if the urine became alkaline, as suggested by Travell, Bodansky and Gold (1940).

Urinary excretion of nicotine in tobacco-smokers was investigated by Wolf and Giles (1950) in 31 adult male habitual tobacco-users, who consumed 15.6-58.4 gm. tobacco during 6.5-8 hours; the amount of nicotine absorbed was found to be 8.0-57.7 mg., and that excreted in the urine 0.55-3.43 mg. Calculated as percentage of the total nicotine dose, the excretion in tobacco-smokers was about the same as that found with cigarette-smoking.

Nicotine has been detected in the urine in cases of acute tobacco or nicotine poisoning in man (Rabot, 1856; Fretwurst and Hertz, 1932; Haverkate, 1937; Moller and Simensen, 1939).

Melner (1944) stated that he had the urine of tobacco-workers analyzed, and that there was every reason to suppose that it contained nicotine. Mokranjac, Radmit and Galijan (1955) estimated the nicotine content of the urine of workers in a tobacco factory; 3 engaged in classification of dry leaves had 3, 6.5, and 8 mg. per liter of urine; 3 engaged in classification of humid leaves had 1.2, 6, and 3; a worker in drying had 2.7; and 2 workers engaged in the production of cigarettes had 5 and 6 mg. per liter.

Concerning factors affecting urinary excretion of nicotine in smokers, F. Cramer (1925b) gave it as his opinion that quick elimination of nicotine might occur in the young smoker who has not smoked long, but that in old smokers a quick elimination of nicotine no longer took place. Burstein (1932) calculated that there were enough blood cells per kg. body weight to adsorb 0.28875 mg. nicotine, and he suggested that this was why doses of this magnitude caused no or only weakly toxic effects, and that urinary excretion of nicotine would depend upon the gradual release of nicotine adsorbed by the blood cells. In this connection, Werle, Schievelbein and Spieth (1956) reported that 4 ml. blood were capable of binding about 0.1 mg. nicotine, this property being independent of age and sex of the blood donor, and equal in smokers and non-smokers. Isar (1951) reported analytical data interpreted as showing that Atabagic (a proprietary preparation) promoted urinary

excretion of nicotine; however, his control studies do not appear to be very adequate.

Dingemans and Freud (1933) isolated from the urine of normal persons a substance which produced signs of catatonia when injected into rats. This substance, which they termed "Catatonin," had the same potency as nicotine in producing catatonia; nicotine and catatonin gave the same blood-pressure effect on decerebrate rats; and the two substances possessed identical chemical properties. The variable amount of catatonin found in the same person, corresponding to whether or not he smoked, was taken as further evidence for the identity of catatonin and nicotine. Lockett (1944) isolated from human urine, both of smokers and non-smokers, as well as from the urine of female dogs, a base which was identified in "final form" as 1-nicotine; the term "final form" was used advisedly, because certain results during the stages of purification of the substance suggested that the original form might not be 1-nicotine. Further work (Lockett, 1946) convinced her that the base was excreted in a form differing from 1-nicotine, but readily converted to 1-nicotine by the simple procedure of subjecting salts of the base to a high vacuum.

#### Feces

Werle and Uschold (1948) found from 6 to 8% of administered nicotine in the feces of rats; they considered that the nicotine might have been excreted in the bile. Following intravenous injection of radioactive nicotine into 3 mice, 1.2% of the total radioactivity was found in the feces 3 hours after injection, and 2.2% 6 hours afterwards (Ganz, Kelsey and Geiling, 1951).

#### Bile

Using dogs under chloralose with bile duct cannulated and (in some of the animals) anastomosed to the jugular vein of a second dog, Hermann and co-workers (1930) found that intravenously injected nicotine was eliminated rapidly and in important quantity into the bile, as judged by the hypertensive reaction on the second dog and by a positive Roussin reaction by the bile. That the fecal excretion of nicotine may be due to biliary excretion has been suggested by Werle and Uschold (1948).

#### Expired Air

Baglioni (1927) claimed that pulmonary excretion of nicotine in the frog stopped when the degree of concentration of nicotine in the air approached that in the organism. In discussing nicotine hyperpnea in the dog, Baglioni stated that it could logically be supposed that, when nicotine was eliminated from the pulmonary surface soon after its presence in the blood traversing the lung, it excited the afferent endings of the vagus and provoked dyspnea by a reflex arc. The evident purpose of this reflex to the defense of the organism was considered to be that of a more rapid and energetic elimination of the alkaloid, the lung thus behaving as an organ of external secretion. E. Adler (1923) reported that a diagnostic clue to the condition he termed *Zigarettenmagen* appeared in the finding that, when the patient had bathed and was lying in a bed with clean linen, although he had not smoked for some days, a peculiar metallic nicotine odor could be noted on deep expiration; such patients were said to be in a state of chronic nicotine intoxication.

The possibility of an appreciable nicotine elimination by excretion into the air cannot be considered seriously, for the volatility of nicotine from dilute solutions is very low (Larson, 1952).

### Skin

According to Baglioni (1927), some nicotine can be experimentally demonstrated to be present in the water bathing a nicotine-injected frog. This diffusion from the cutaneous surface was held to constitute a process of elimination of the poison; it stops when, in the external environment, the degree of concentration approaches that in the organism.

No reliable data are available regarding excretion of nicotine in the perspiration, although there is no reason to doubt but that the body-fluid concentration of nicotine is reflected in some proportion in any perspiration that forms (Larson, 1932). Several clinical reports have appeared on this subject. A man, an inordinate user of tobacco and exposed as well for several hours daily to the fumes arising from tobacco while being "stoved," was taken ill, and during hospitalization, it was noted that a strong smell of nicotine was present in his perspiration (Babington, 1866). Vapor baths were started, and it was stated that, when in the bath, the other patients in the ward all recognized the odor of tobacco. The pajamas of an individual who smoked more than customarily, and who perspired heavily during the night, were washed, and 0.48 mg. nicotine found in the washings (Bodnar and Novak, 1934). Tittmeyer (1937) called attention to the observation that, in physical medicine, large amounts of nicotine are found in the wet dressings applied to smokers. "We put him into hot packs," wrote Wharton (1946) of a man suffering from terrific headaches and treated by "elimination," "keeping him there till perspiration had been established and had had time to eliminate all the skin could at one time. How the nicotine did come out! It filled not only the blankets but the room with its odor. The patient's headache naturally disappeared.

### Milk

Kosliak (1974) noted that the milk of nursing female cigar-makers had an odor of tobacco. Identification of nicotine in milk has been largely by biological rather than by chemical techniques. Woller (1927a, b) considered that the then-existing methods for determining nicotine in milk were not sensitive enough to detect the substance in woman's milk or in that of nicotineized animals.

Hatcher and Crosby (1925) reported an experiment on a young woman 5 days post-partum who had smoked 20-25 cigarettes daily for 6 days. After smoking 7 cigarettes in 2 hours, about 26 ml. of milk were obtained and extracted; the total amount of nicotine in the extract did not exceed 0.015 mg., and 40% of the extract injected into a frog caused only a feeble twitching of a few muscles.

In a study by Emanuel (1931), in which 10 nursing mothers smoked 6-15 cigarettes in 1-2 hours, milk collected at 2-3, 4-5, and 7-8 hours after smoking and assayed on the leech-muscle preparation was found to contain nil to 0.03 mg. of nicotine per liter. The presence of nicotine was encountered more frequently during the 4-5 hour period, and more frequently and in greater quantity with inhalers than with non-inhalers.

Tonn (1932) obtained a positive Roussin reaction for nicotine on 5 ml. of mother's milk.

Using a modification of the method of Hatcher and Crosby (1925) for extracting nicotine from milk, and a frog test for bio-assay, W. B. Thompson (1933) obtained positive tests for nicotine on milk from 4 mothers, while in 3 other cases, the results were classed as questionable. In no case was a chemical test for nicotine positive, and the amount of nicotine extracted from the milk must have been exceedingly small,

since, in most cases, all or most of the extract was injected into a single frog.

Nagy (1934a) was unable to demonstrate nicotine in the milk from a woman who smoked 15-20 cigarettes daily; even when 25-30 cigarettes were smoked in half a day, only 0.013-0.015 mg. nicotine per liter could be found. This was the maximum quantity found in the heaviest smoker of 3 women, each of whom smoked 15-30 cigarettes; the other two secreted no perceptible quantity (Nagy, 1934b). The nicotine was distilled from the milk and precipitated with silicotungstic acid.

Bisdorf (1937) stated that he had found nicotine in the milk of a nursing mother who smoked an average of 60 cigarettes a day; the milk caused signs of nicotine intoxication in the nursing.

Perlman, Dannenberg and Sokoloff (1942) used *Daphnia magna* for the bio-assay of nicotine in milk. Smokers of 1-4 cigarettes daily gave an average excretion of 0.116 mg. per liter in morning specimens and 0.16 mg. in afternoon specimens. The corresponding figures for those smoking 5-10 and 11-20 cigarettes daily were 0.225, 0.276, and 0.445, 0.5 mg. per liter. Urinary concentrations of nicotine were found to be 11-17 times greater than that of the milk.

The work of Hatcher and Crosby (1925), Emanuel (1931), and Perlman, and co-workers (1942) has been briefly reviewed by J. H. Burn (1947). Sapeika (1947) has reviewed the literature on the subject of the excretion of nicotine in milk.

Two experiments on cows by Hatcher and Crosby (1925) gave the following results: a 455-ml. milk sample taken 1.5 hours after intramuscular injection of 2.153 gm. of nicotine in 4 divided doses over a 3-hour period contained an estimated 0.1 mg. of nicotine. A specimen (of similar size) taken 5 hours after injection of 200 mg. contained only a trace of nicotine.

### DETOXICATION (METABOLISM) OF NICOTINE

The foregoing studies on the excretion of nicotine indicate that only a small percentage of absorbed nicotine is excreted unchanged by the body. The major portion of absorbed nicotine is detoxified within the body.

### Sites within the Animal Body

As long ago as 1876, evidence was noted for the detoxification of nicotine by the liver. B. F. Lautenbach (1876-77) found that injection of 1 drop of nicotine subcutaneously or into a vein of the general circulation of a dog was fatal, whereas 1 drop injected into the mesenteric or splenic vein, so that the drug had to pass through the liver before it could enter the general circulation, produced only mild poisoning with prompt recovery. In the normal dog,  $\frac{1}{5}$  drop of nicotine never produced death, but in dogs whose vena portae had been tied, death usually occurred rapidly when but  $\frac{1}{5}$  drop was injected. In normal frogs,  $\frac{1}{20}$  drop of nicotine always produced marked symptoms of poisoning, but never death; in hepatectomized frogs, even  $\frac{1}{40}$  drop produced death. On the other hand, when the liver of the frog was rendered hyperemic by tying off the superior vena cava to force all the venous blood of the abdomen to flow through the liver,  $\frac{1}{50}$  or  $\frac{1}{5}$  of a drop of nicotine (the fatal dose, and twice the fatal dose for a frog) was no longer fatal; the greater activity of the liver was believed to lead to destruction of a larger quantity of the nicotine.

Although Lautenbach (1870-80) later abandoned the idea that the liver specifically destroyed nicotine, since his results following injection of nicotine into the femoral artery of cats and rabbits were precisely similar to those earlier observed

following injection into the portal vein of dogs, from which he concluded that compelling nicotine to pass through any set of capillaries seemed to prevent its being poisonous, the detoxifying power of the intact liver has been repeatedly confirmed by later workers employing a variety of techniques. Rothberger and Winterberg (1905) pointed out that larger doses of nicotine, among other substances, could be taken by mouth than by subcutaneous injection, which might indicate the possibility of a specific antitoxic function of the liver. Using Eck-fistula dogs, these authors tested this point with atropine, administering one-half the subcutaneous lethal dose by stomach tube to 5 animals and to 3 normal controls; the controls all recovered, while the 5 fistula dogs all died from atropine poisoning. Fleig and de Visme (1907c) also noted in dogs that intra-gastric or portal vein injection of tobacco-smoke solutions failed to produce cardiovascular effects even in doses infinitely stronger than those producing effects on intravenous injection. In the intact frog, Schulmann and Egert (1917) found that subcutaneous injection of nicotine was more rapidly lethal than intestinal injection of the same doses. This sequence was found to be reversed in frogs hepatectomized following ligation of the hepato-biliary pedicle, thus demonstrating the antitoxic action of the liver to nicotine. Biebl, Essex and Mann (1932) found that, whereas injection of 10 mg./kg. nicotine into the dorsal lymph sac of 36 normal frogs was not fatal, injection of the same dose into 15 totally hepatectomized frogs was fatal to 10, and recovery of 4 of the remainder took 2-3 times as long as in the controls. In 6 partially hepatectomized frogs, 3 died and recovery was somewhat delayed in the surviving 3. These authors also perfused heart-lung, heart-lung-liver, and heart-lung-hind limb preparations of dogs with blood containing initial concentrations of 0.1 mg. nicotine per ml. In the heart-lung preparation, the amount of nicotine in the circulating blood did not show any significant change over a period of 2 hours, and in the heart-lung-hind limb preparation, there was a sharp fall in nicotine concentration to about half its original value in 15 minutes, after which it remained constant for the duration of the experiment, the initial drop being apparently due to diffusion. In the heart-lung-liver preparation, however, blood samples coming from the liver 15 minutes after the introduction of nicotine showed a large diminution in nicotine concentration, and successive samples showed less and less, until 70 minutes after the introduction of nicotine, the assay of a blood sample indicated no appreciable nicotine present. In dogs, following an injection of 1 mg. nicotine, there was a moderate pressor response lasting about 1 minute. After the liver was removed, the response to a proportionate dose (0.95 mg.) was more pronounced and lasted 3 minutes. Mice in which liver damage had been produced by carbon tetrachloride tolerated repeated nicotine administrations poorly, as compared to normal control animals (Haag and Larson, 1944; Haag, Larson and Finnegan, 1945). If the liver of guinea pigs was damaged by phosphorus poisoning, its detoxifying power was found to drop about 70%, while that of the lung and kidney was almost unaffected (Werle and Uchold, 1948).

In contrast to the preceding authors, Heubner and Papierkowski (1938) concluded that their experiments showed no great influence by the liver in detoxifying nicotine. They gave 20 mice 0.4 mg. nicotine tartrate orally, and 20 animals the same amount subcutaneously, every half hour (equivalent to 24 or 35% of L.D.<sub>50</sub> per dose). By this procedure, the amount necessary to kill was increased 2-3-fold, but those animals receiving nicotine by mouth tolerated more nicotine than those receiving it subcutaneously, in the ratio of 4:3.

In connection with the ability of the intact liver to detoxify nicotine, Takahashi (quoted by Hofstätter, 1934a) stated that tolerance to nicotine could be increased by the use of glycogen, either before or along with the nicotine. Hambresin and Schepens (1946) stated (without, however, adducing proof) that the ability of the liver to detoxify nicotine varied directly with its glycogen content. In the presence of an alkaloid such as nicotine, the hepatic glycogen was supposedly transformed into glucose, which united with the toxic substance, and an oxidation transformed the complex thus formed into a conjugated glucuronic acid.

As indicated above, Lautenbach (1879-80) believed that the hind limbs, via their capillary beds, were able to detoxify nicotine, but that this apparent detoxication might be due to diffusion is suggested by the work of Biebl, Essex and Mann (1932) on the heart-lung-hind limb preparation, described above.

Apparently, no significant detoxication of nicotine occurs *in vivo* in the lung of the dog (Biebl, Essex and Mann, 1932).

As judged by perfusion experiments with isolated guinea pig hearts using radioactive nicotine, it appears that the isolated heart muscle of the guinea pig did not possess the ability to metabolize nicotine to any appreciable extent (Ganz, Kelsey and Geiling, 1951; Geiling, 1951). However, the time element and quantitative aspects of this study make it difficult to evaluate.

Alessio (1925) introduced nicotine into rectal and sigmoidal loops (caudal and portal circulations) of rabbits. In doses somewhat larger than those needed to produce hypotension when given intravenously, the nicotine introduced into the intestinal loops had no effect on blood pressure, from which it was concluded that the drug was probably inactivated in the cells of the colonic mucosa.

#### *Experiments on Tissue Breis, Slices, Homogenates, Extracts*

Lautenbach (1876-77) macerated livers of dogs and rabbits with nicotine, then injected the expressed juice from the macerated mass into dogs and frogs; and he noted that, under these circumstances, fatal symptoms of nicotine poisoning were never produced. In control experiments, kidneys of healthy animals were macerated with nicotine, and the expressed juice produced death of the animals into which it was injected; the expressed juice of the kidney itself was not toxic. Wenusch (1935b) incubated 2 batches of 100 gm. of fresh pig-liver brei to which 22 mg. nicotine had been added; one batch was allowed to incubate for 5 hours, the other for 24 hours at 37°C., following which 22 mg. nicotine were recovered from the 5-hour sample and 21.5 mg. from the 24-hour sample. Wenusch concluded that the liver played no role in the detoxication of nicotine, and that loss through other secretions or alterations of the pyridine ring must account for the discrepancy between injected nicotine and that excreted in urine. Werle and Becker (1942) confirmed that tissue brei made from liver by grinding with sand showed no nicotine destruction, a finding which was perhaps explicable by a quick autolytic destruction of the detoxicating enzyme. Werle and Uchold (1948) demonstrated on human tissues that the nicotine-detoxifying enzyme was much less active in organ breis; this was not due to destruction of cell structure, but to autolytic processes. When liver brei was added to liver slices, the detoxifying capacity of the slices was reduced by one-half, or completely abolished if greater amounts of liver brei were used.

Werle (1938) took exception to the work of Wenusch (1935b) on hashed liver, and used the Warburg technique to

study the detoxifying power of tissue slices. The technique used was that of incubating freshly prepared tissue slices of various organs in Tyrode's solution containing nicotine, the amount of nicotine detoxified being determined by the amount not recoverable by distillation and colorimetric determination after a suitable time period. Table 1-4, embracing the findings of Werle and his co-workers, has been assembled from their various publications. It will be seen that, varying somewhat with the species of animal studied, liver, lung, kidney, and brain may be sites of detoxification of nicotine. Other tissues, such as muscle, spleen, small-intestine mucous membrane, adrenals, skin, and blood, studied in some of the species, failed to detoxify nicotine. Nicotine detoxication by the organs of a species appeared not to have a direct connection to its nicotine sensitivity; thus, the liver of the rabbit and the pigeon detoxified nicotine about equally, whereas the sensitivity of the pigeon to nicotine was very great compared to the rabbit; and the lung of the pigeon, in contrast to that of the rabbit, showed no detoxication capacity for nicotine. A. W. Miller, Jr. and Larson (1953), also using the Warburg technique, found that, for a given species, liver metabolized nicotine more rapidly than any other tissue (Table 1-5). In order of decreasing ability to metabolize nicotine, the other tissues studied possessing this property showed no consistency from one species to another. The marked species variation in the ability of intact animals to metabolize nicotine cannot at present be satisfactorily explained by the rates at which their tissues detoxify the alkaloid *in vitro*.

Werle and Müller (1941) emphasized that studies on detoxication of nicotine must be done on organs removed and used as soon as possible from the killed and bled animals, since their detoxication capacity rapidly decreased. Organs kept 24 hours at 15°C. showed no or only slight detoxication capacity; this appears to explain why organs from human corpses show no detoxication capacity.

Yamamoto, Kuroguchi and Takeuchi (1955) found that

TABLE 1-4

Relative Rates of Detoxication of Nicotine by Tissues from Several Species of Animals

(Assembled from data of Werle, 1935; Werle and Müller, 1941; Werle and Becker, 1942; Werle and Uchold, 1948)

Species	Per Cent of Added Nicotine Detoxified by:									
	Liver	Lung	Kidney	Brain	Muscle	Spleen	Small intestine	Yucca (blood)	Placenta	Adrenal
Rabbit	100	ca. 40	ca. 15	0	0	0	0			
Sheep	100	ca. 60	0	0	0	0	0			
Pigeon	80	0	ca. 25	0	0	0	0			
Guinea pig	57	26	17	0	0	0	0			0
Dog	40	17	0	0	0	0	0			
Cat	40	16	10							
Man	35-42	25-30	10-25	10-16	0	5-6	0-7	0	15-20	
Rat	30	14	5-8	10	0	0	0			
Pig	±	0	0	0	0	0	0			
Cattle	0	0	0	0	0	0	0			

\* Based on adding 0.4 or 0.5 mg. of nicotine in 0.5 ml. of water to 0.8 gm. of tissue slices in 3 ml. Tyrode's solution and incubating under oxygen for 3 hours at 37°C.

TABLE 1-5  
Species Ability to Detoxify Nicotine  
(Adapted from Miller and Larson, 1953)

Species	Ability to Detoxify Nicotine*							
	Liver	Lung	Kidney	Spleen	Muscle	Small intestine	Adrenal	Placenta
Mouse	+	+	+	±	0		0	0
Rabbit	+	+	+	0	+	+	+	0
Cat	+	+	+	+	+	+	+	0
Dog	+	+	+	+	+	+	+	0

\* Based on adding 0.06 mg. nicotine to 60-80 mg. of tissue slices in 4 ml. Tyrode's solution and incubating under oxygen for 3 hours at 37°C.

organ extracts, prepared by homogenizing fresh organs of the rabbit with 2.5% Ringer's solution followed by centrifugation, detoxified nicotine in the following relative proportions: liver extract, 100; lung extract, 45; kidney extract, 30; blood plasma, 36.

Tsujiimoto (1957) stated that he had demonstrated nicotine oxidation by tissue homogenate by manometric means in the livers and kidneys of dogs, pigs, and cats, and in the livers of rabbits, guinea pigs and horses; nicotine oxidation did not take place in liver homogenates of cattle, albino rats, and mice. Nicotine oxidation by rabbit liver and nicotine oxidation by rabbit liver and kidney was also observed. The activity of the nicotine oxidation was placed in the mitochondria of rabbit liver. Hucker (1938) found that nicotine was metabolized in the 9000 × g fraction of rabbit-liver homogenates (for further details, see the following section).

#### Nature of the Detoxication Process

From the studies of Werle and his colleagues referred to, the following information concerning the nature of the detoxication reaction has appeared: The reaction is dependent on the intact cell; grinding the organs in a mortar with sand, or running them several times through a meat-grinder, destroyed their detoxication capacity (Werle and Müller, 1941). This was not due to destruction of cell structure, but to autolytic processes (Werle and Uchold, 1948). After freezing in carbon-dioxide snow, the detoxifying power of liver slices was completely lost. Brief warming of liver slices to 56°C. destroyed its fermentative action (Werle and Koebke, 1949); by short boiling in Tyrode's solution, the detoxication ability of lung increased, while that of liver was reduced about 70% (Werle, 1938). When slices of liver are extracted by washing with Tyrode's solution, 10% sodium chloride solution, or distilled water, they lose their detoxication activity more or less completely, dependent on the duration of suspension; and addition of the washed liquid to the slices does not restore their activity (Werle, Schievelbein and Spieth, 1956). The detoxication reaction is dependent on the presence of oxygen (Werle, 1938), and proceeds optimally at neutral pH, being nil at pH 6 but still effective in alkaline media at pH 9 (Werle, 1938). It does not proceed in an atmosphere of nitrogen (Werle, 1938; Werle and Müller, 1941), and it can be inhibited by carbon monoxide and potassium cyanide (Werle, 1938), and by chloroform, sodium azide and methylene blue (Werle and Müller, 1941), but not by hydroxylamine (Werle and Müller, 1941).

or by semicarbazide (Werle and Müller, 1941; Werle, Schievelbein and Spieth, 1956). From his early studies with cyanide and carbon monoxide, Werle (1935) concluded that an enzyme was involved. From the fact that carbonyl group compounds, such as hydroxylamine and semicarbazide, had no effect on nicotine detoxication, Werle and Müller (1941) concluded that the inhibition by hydrocyanic acid related, probably not to the blocking of a carbonyl group, but to blocking of heavy metal atoms of the active group of the enzyme. Metal-complex formers, such as hydrocyanic acid, phenylthiourea, sodium pyrophosphate, 6-hydroxy-quinoline, dithione, and trilon inhibit the reaction, which was considered as proof that a heavy metal enzyme was participating in the detoxication process (Werle, Schievelbein and Spieth, 1956). Copper sulphate caused a barely significant inhibition (Werle and Müller, 1941). Addition of the plant proteinase, papain, reduced the detoxifying power of liver slices by about 50% (Werle and Uebachs, 1948). Addition of methionine to the liver slices reduced the rate of detoxication of nicotine about 60% (Werle and Uebachs, 1948). The metabolism of nicotine was completely inhibited in the presence of nicotine, although the metabolism of nicotine was not affected by nicotine (Werle and Koeber, 1949). The breakdown of nicotine by liver slices was inhibited by isonicotinic acid (Schievelbein and Werle, 1957).

Werle, Schievelbein and Spieth (1956) considered that loss of detoxication capacity by liver homogenates could be conditioned by the fermentative destruction of the carrier proteins of the ferment system to be detoxicated (e.g. by cathepsin), by a loss of SH groups in the ferment system, or by the loss of adenosinetriphosphoric acid (ATP), which could be connected with the nicotine decomposition, by the activation of ATP-ase in the homogenate. They therefore investigated whether the decomposition capacity for nicotine in the homogenate remained intact in the presence of glutathione, cysteine, ascorbic acid, ATP, or sodium fluoride, the last-mentioned of which impairs the decomposition of phosphate compounds with in energy; these experiments gave negative results. Since structure-bound oxidation systems are mainly located in the mitochondria, a preparation of liver mitochondria was obtained by fractional centrifugation by the method of Hogeboom and Schneider (J. Biol. Chem. 172: 619, 1948); but this preparation was not capable of detoxifying nicotine.  $\gamma$ -Di-nitrophenol, breaking up the phosphorylation of atomic chains, considerably inhibited nicotine detoxication. Addition of adenosinetriphosphoric acid or phosphoglyceric acid had no effect. Succinic acid as a component of the citric-acid cycle was without effect, i.e. the decomposition of nicotine does not interfere with the citric-acid cycle. Cysteine, glutathione, and ascorbic acid did not activate the decomposition of nicotine, nor regenerate the lost decomposition capacity of aged slices of liver, leading to the conclusion that no functionally effective SH groups are present in the enzyme system which could be protected or regenerated by these substances. An observed inhibition by moniodide acetic acid was held to be ambiguous, since some dehydrogenases are strongly inhibited by the substance. Magnesium ions effected a weak activation, manganese ions a weak inhibition; accordingly, it was thought that  $Mg^{++}$  could be a co-factor, and that it is possible that the inhibition by manganese consisted in the removal of  $Mg^{++}$ . Addition of aneurine and thiocyanate had no effect. Snake venom inhibited considerably, probably because it attacks the structure of the liver slices. Several antihistaminics inhibited strongly *in vivo*, but not when tested *in vitro*.

Slices of liver suspended in liver "broth" (prepared by heat-

ing to 70°C. a liver homogenate prepared with 3 parts water) detoxified more nicotine than when suspended in Tyrode's solution (Werle, Schievelbein and Spieth, 1956). If the homogenate from which the broth is prepared is alkalized with magnesium oxide before heating, the yield in activator is increased. The active principle can be precipitated with ammonium sulphate, and is insoluble in alcohol. It is not dialyzable through cellophane. It is lost by concentrating the broth *in vacuo*. It cannot be detected in the distillate nor in the residue. The distillate of the broth, when collected in acid, inhibits nicotine decomposition. The "inhibitor" is readily volatile. It is not identical with ammonia, methylamine, ethylamine, dimethylamine, trimethylamine, propylamine, dimethylaminoethanol, choline, acetylcholine, or hydrogen sulphide. It was thought possible that the inhibitor is a decomposition product of choline, since, when choline chloride is treated with alkali in the Conway preparation, a substance goes over into the acid used which inhibits the nicotine decomposition by liver slices.

Hucker (1956) found that nicotine is metabolized in the  $9,000 \times g$  fraction of rabbit-liver homogenates. Triphosphopyridine nucleotide (TPN) and oxygen were required for the reaction. Neither microsomes nor  $78,000 \times g$  supernatant alone would oxidize the alkaloid, but activity was restored on recombining the cell components. It was found that the role of the supernatant is to maintain TPN in the reduced form while the nicotine oxidation occurs in the microsomes. The system was inhibited by cytochrome C, methylene blue, and  $\beta$ -diethylamino-ethyl-diphenylpropyl acetate (SKF 325-A). Sodium cyanide reversed the inhibition produced by cytochrome C. Neither diethyldithiocarbamate nor glutathione had a significant effect on the reaction.

The liquid in which liver slices were suspended can detoxicate a considerable amount of nicotine (Werle, Schievelbein and Spieth, 1956). The detoxication ferment itself is not concerned in this effect, for the nicotine disappears instantly after admixture, and the amount detoxified does not increase with the duration of the reaction, as compared to the fermentative decomposition. Thus, a substance is extracted from slices of liver by washing which binds nicotine instantly, so that the König reaction, employed for its determination, is negative. Nicotine will not be liberated again from the reaction solution, either by boiling with alkali or with acid. The amount of nicotine-binding substance was not altered by suspension of liver slices in physiological potassium-chloride solution, or by the addition of hyaluronidase or lysolecithin to the suspension preparation. Liver broth showed equal nicotine-binding capacity. Blood also binds a slight amount of nicotine, and slices of placenta washed blood-free also possessed a binding capacity for nicotine. The amount of nicotine bound varied with the animal species from which the liver was obtained. Using 0.8 gm. slices plus 0.4 mg. of nicotine, about 14% of the nicotine was bound instantly. Initially, the amount of nicotine bound increases distinctly with increasing duration of incubation; then, the nicotine is gradually completely liberated. Binding and liberation take place only in an oxygen atmosphere, and not in an atmosphere of nitrogen. The product liberated may represent a decomposition product of nicotine.

Yamamoto, Kuroguchi and Takeuchi (1955) reported that nicotine-detoxicating ability was found in blood plasma and in extracts of rabbit liver, lung, and kidney, liver extract being the most potent. When a 25% extract of rabbit liver (25% homogenate in Ringer's solution, centrifuged, upper clear layer used) was added to 3 mg. of nicotine per ml., and the mixture stirred at 37.5°C., determination of nicotine by the

silicotungstic acid method or by bio-assay on isolated guinea pig intestine indicated that it decreased during the 3-hour incubation period, but determination by the CNBr method showed no change of nicotine content (Takeuchi, 1955). As determined by the silicotungstic acid method or by bio-assay, addition of potassium cyanide or sodium azide, or heating at 56°C. for 30 minutes, inhibited the decomposition of nicotine in the extract. Using guinea pig liver slices, Werle and Meyer (1950) found that 10-15 gamma nicotine in a volume of 2.5 ml. increased oxygen consumption, while 5-7.5 mg. decreased it; they concluded that a stoichiometric relation between oxygen use and nicotine metabolism could not be arrived at. Takeuchi (1955) stated that oxidation of nicotine in the system: rabbit liver extract-nicotine had been demonstrated by manometric methods, as did also Tsujimoto (1957) for kidney and/or liver homogenates from several species. As determined by the Thunberg method using methylene blue, nicotine was claimed to be dehydrogenated by rabbit liver extract; the dehydrogenation was not influenced by potassium cyanide (Takeuchi, 1955).

Studies on the intact dog, excluding nicotine excreted unchanged in the urine, have shown that rate of detoxication within the body bears a logarithmic relation to nicotine concentration (Larson, Larson and Haag, 1954). This was also shown to be the case for guinea pig liver slices (Werle and Uschold, 1948).

A number of investigators have studied the effect of development of tolerance on the rate of detoxication of nicotine. Dixon and Lee (1912) injected rabbits with nicotine subcutaneously or intravenously on alternate days until 15 injections had been given, then 30 days after the last injection, killed the animals and prepared extracts (by grinding the tissues in sterile sand) of liver, brain, spinal cord, and striated muscle. In the great majority of experiments, the amount of free nicotine (bio-assayed by blood-pressure response of decerebrate cats) contained in tissue-extract solutions after incubating with nicotine at 35°C. for 2.5 hours was greater in extracts from normal control (litter-mate) rabbits than in extracts made from nicotine-injected animals. Boiling the liver before incubation greatly reduced the degree of inactivation of nicotine. Expressed liver juice gave the same result as liver extract. Since loss of activity proceeded only very slowly, the authors felt they could rule out the possibility that nicotine combined with some constituent of the tissue extract present in larger quantities in the tolerant animal which rendered the alkaloid inactive, and they considered the evidence indicated that the detoxication process was possibly in the nature of a ferment action. In confirmation of this latter view, dried samples of liver gradually lost their ability to detoxify nicotine in about 9 days. C. W. Edmunds and Smith (1915-16a, 1916b) undertook to repeat the work of Dixon and Lee using the livers of dogs. Ten dogs were rendered tolerant to nicotine in the manner described by Edmunds (1909), and when the process was complete, the animals were killed and the ground liver incubated with a definite amount of nicotine, nicotine being subsequently determined by bio-assay on blood pressure of the cat. In 4 dogs, there was no difference in nicotine-destroying power between the nicotine-injected animals and normal controls; in 3, the liver from the tolerant animal destroyed more than did the normal control, but this was offset by the remaining 3 of the 10 dogs in which the controls were more active than the tolerant livers. Takeuchi, Kuroguchi and Yamaoka (1954) prepared liver extracts from rats given daily intramuscular injections of 5 mg./kg. nicotine for 30-120 days (this dose sufficed to produce convulsions following each in-

jection), and found no significant differences between extracts of livers of intoxicated animals and those of controls in ability to detoxify added nicotine. Werle and Müller (1941) studied the detoxifying power of liver slices from rats chronically injected with initially convulsive doses of nicotine (5 mg./kg. subcutaneously) over a period of 30-60 days. Contrary to their expectations, no increase in nicotine detoxication rate was found; in fact, in contrast to untreated animals, detoxication was even decreased. Werle and Uschold (1948) repeated the experiments using rats receiving a constant nicotine dose of 0.005 mg./gm. every other day for 8 weeks, and then every fourth day for 4-6 months in 1 group of 6 animals, and in a second group of 6 animals receiving 0.005 mg. nicotine per gm. increasing by 0.001 mg./gm. every 8th and later every 12th day; each nicotine-injected group was accompanied by 2 Ringer-injected controls. Results showed that the detoxifying capacity of liver was increased only a little (37% and 38% detoxication, compared to 31% in the controls), while that of lung, kidney, and brain increased 100% over the controls, the increases being about equal in the animals from both dosage regimens. The authors concluded that the increased detoxication capacity for nicotine, developed through its chronic administration, was limited, at least in the rat.

If the liver of guinea pigs were damaged by phosphorus poisoning, its detoxifying power was found to drop about 70%, while that of the lung and kidney was almost unaffected (Werle and Uschold, 1948).

Although rats receiving intramuscular injection of 30 mg. ascorbic acid daily for 10 days were more resistant to nicotine injection, the detoxication capacity of their livers for nicotine was not noticeably increased (Werle, Schievelbein and Spieth, 1956).

#### Nature of the Products of Metabolism

The structural formulas of some of the pyridine derivatives mentioned below are given in Figure 1-1 [taken from Larson (1952)].

Ganz, Kelsey and Geiling (1951) found that all or almost all of the radioactivity from  $C^{14}$ -randomly-labeled nicotine, administered to rats, was excreted in the urine in 16 hours; and Bennett, Tedeschi and Larson (1954) found about 95% of the activity in the urine within 36 hours after administration of similar material to dogs. Clearly then, urine constitutes the chief repository for metabolites of nicotine.

The metabolism of nicotine in the body appears to be quite complex. Owen and Larson (1955) administered  $C^{14}$ -randomly-labeled nicotine to dogs, and by paper chromatography of the urine obtained 7 peaks of radioactivity, indicating the presence of 3 major and 4 minor metabolites of nicotine, in addition to a peak representing unchanged nicotine. From a similar study on the cat, it appeared that there were quantitative and possibly qualitative differences between the nicotine metabolites in this species, as compared to the dog, but that the number of metabolites involved may be comparable. Information that has been gained concerning the chemical nature of nicotine metabolites is given below.

Early studies on nicotinic acid indicated an increased excretion of this substance (Covello, 1939; L. J. Harris and Raymond, 1939), as well as of trigonelline (Linnweb and Reinwein, 1932b; Melnick, Robinson and Field, 1940a, b), in the urine of smokers. Evidence that these results were due to the non-specific character of the analytical procedures used, excretion of unchanged nicotine being reflected in them, soon appeared (Perlzweig, Levy and Sarett, 1940, personal communication from Dr. Perlzweig; H. Field, Jr., P. P. Fox and



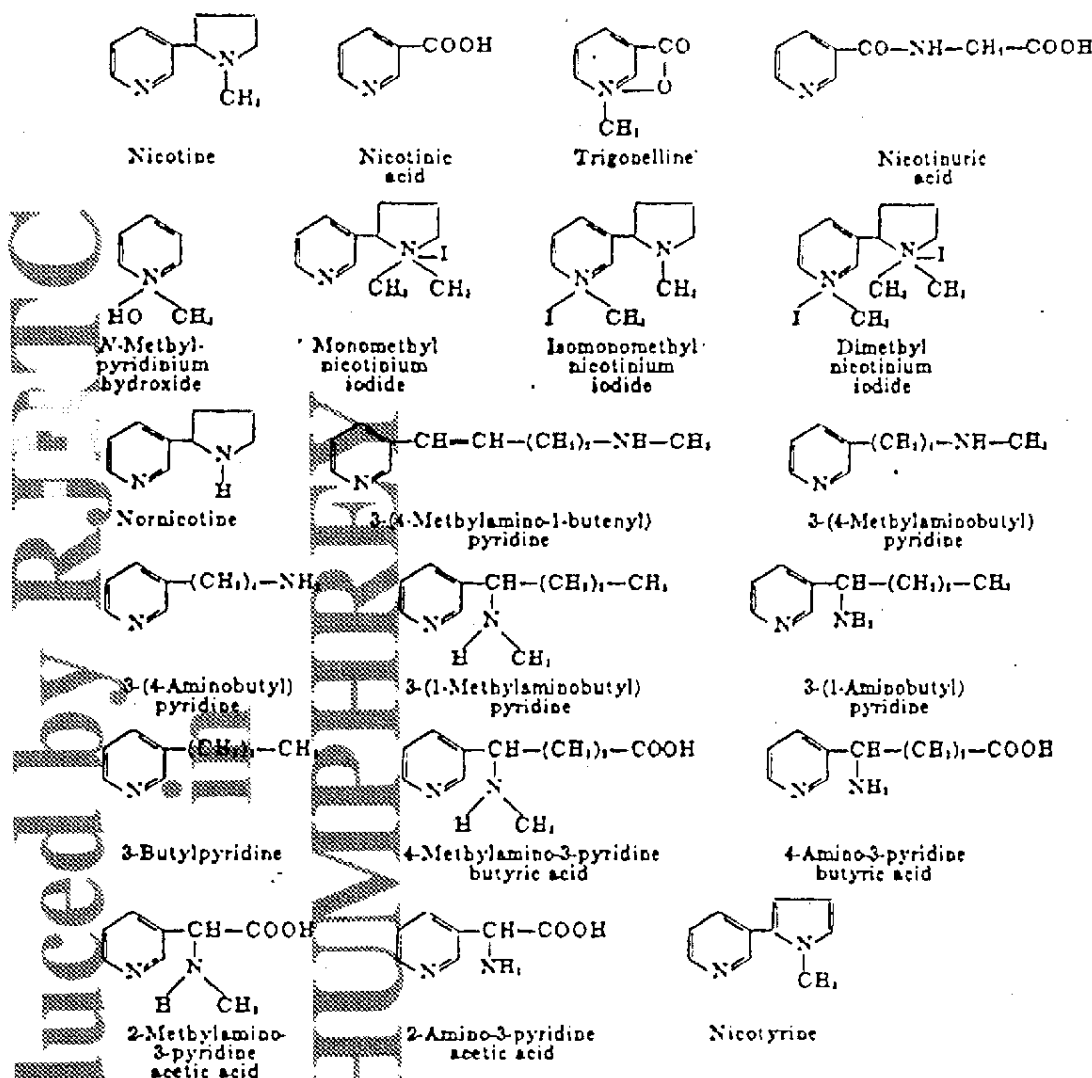


FIG. 1. Structural formulas of some of the pyridine derivatives mentioned below. (From Larson, 1952, courtesy of Industrial and Engineering Chemistry.)

N. L. Fot, 1946). In an experiment on the dog, designed to study the effect of administered nicotine on the urinary excretion of nicotinic acid, nicotinuric acid, trigonelline, and N-methylpyridinium hydroxide (a metabolite of pyridine, Tomita, 1921). Larson and Haag (1942a, b) found no increase in the excretion of these compounds over the control periods. Similarly, studies on the formation of nicotinic acid, nicotinamide, trigonelline, and N-methylpyridinium hydroxide from detoxication of nicotine by liver slices gave negative results, and no steam-volatile basic pyridine compounds were found (Werle, Koebeke and Meyer, 1950). It would appear, then, that metabolism of nicotine to nicotinic acid or pyridine does not occur, or, if it does, the amounts formed are exceedingly small.

In the study on dogs referred to above, Larson and Haag (1942a, b) noted that, following nicotine administration, the

urine contained a compound yielding a red color when reacted with cyanogen bromide. Control urine did not contain this material, and the reaction of nicotine with cyanogen bromide does not give this color. A metabolite of nicotine giving a red color with cyanogen bromide was also detected by Werle, Schievelbein and Spieth (1956) in the urine of smokers, whereas when cyanogen bromide was added to the urine of non-smokers, a reddish-yellow dye was produced. Both coloring matters could be extracted from urine by shaking with ether and amyl alcohol; that from the urine of smokers showed maximum absorption at 510 mμ, whereas that from the urine of non-smokers showed maximum absorption at 470 mμ.

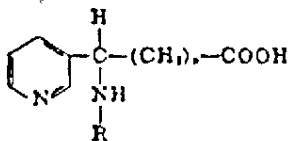
In the dog, the course of metabolism of methylated derivatives of nicotine did not appear to be the same as that of nicotine (Larson and Haag, 1943). Monomethyl-, isomonomethyl-, and dimethyl-nicotinium iodides did not give a red



color when reacted with cyanogen bromide. While normicotine yielded a red color with cyanogen bromide, this substance could be extracted from alkalized urine by ether, whereas the corresponding metabolite from nicotine could not. Larson and Haag (1943) concluded that no evidence had been found to indicate that the detoxication of nicotine in the animal organism involved either methylation or demethylation of the molecule. In a later study, Hucker (1955) found no formaldehyde formed during the metabolism of nicotine by the 8,000  $\times$  *p* fraction of rabbit-liver homogenates, indicating an absence of conversion to normicotine in this system. With respect to methylation of nicotine during detoxication, Werle and Uschold (1948) found that addition of methionine to liver slices being incubated with nicotine depressed the rate of detoxication.

Production of a red color in the reaction between normicotine and cyanogen bromide (see above) led Larson, Haag and Finnegan (1946) to an examination of the behavior of products of cleavage of the pyrrolidine ring of the nicotine molecule. 3-(4-methylamino-1-butenyl)pyridine, 3-(4-methylaminobutyl)pyridine, and 3-(4-aminobutyl)pyridine, representative of products of cleavage of the pyrrolidine ring between the nitrogen and the 2 position, did not yield a red color when reacted with cyanogen bromide. 3-(1-Methylaminobutyl)pyridine and 3-(1-aminobutyl)pyridine, representative of products of cleavage of the pyrrolidine ring between the nitrogen and the 5 position, did yield a red color with cyanogen bromide, but 3-butylpyridine did not. It therefore appears that nicotine derivatives that produce a red color when reacted with cyanogen bromide are limited to primary and secondary amines having the nitrogen substituted on the carbon alpha to the pyridine ring. The nicotine metabolite producing a red color with cyanogen bromide would then represent a product of cleavage of the pyrrolidine ring between the nitrogen and the 5 position. Since the metabolite is not extractable with ether from alkaline urine, the possibility of a carboxyl group at the end of the resulting side chain was immediately apparent. Conceivably, 4-methylamino-3-pyridine butyric acid, 4-amino-3-pyridine butyric acid, and, through beta oxidation, 2-methylamino-3-pyridine acetic acid and 2-amino-3-pyridine acetic acid would all satisfy these conditions, and all became possible nicotine metabolites. Noteworthy for subsequent identification studies (see below) was the additional observation that urine failed to give the color reaction if boiled prior to the addition of cyanogen bromide (Larson and Haag, 1942b).

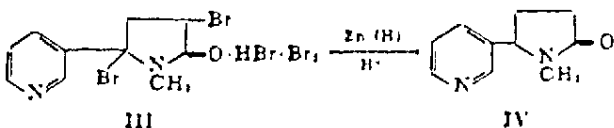
Consideration of the properties of the metabolite(s) responsible for the cyanogen bromide reaction and the behavior of model substances led McKennis and collaborators to the conclusion that the compound in question was probably  $\gamma$ -(3-pyridyl)- $\gamma$ -methylaminobutyric acid (I) or  $\gamma$ -(3-pyridyl)- $\gamma$ -aminobutyric acid (II) [note change in nomenclature to conform to author usage].



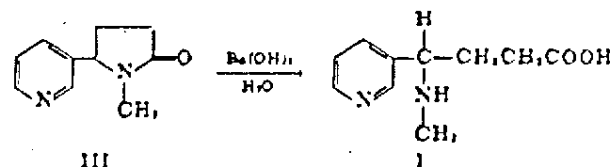
I R = CH<sub>3</sub>, n = 2; II R = H, n = 2.

Both of these compounds, prepared by synthesis (McKennis et al., 1958), give the characteristic red color in the cyanogen bromide reaction before, but not after boiling, which causes lactam formation. The methylamino acid was prepared from

the intermediate dibromocotinine hydrobromide perbromide (III) which was converted to cotinine (IV); method of Pinner (Ber. 26: 292, 1893):



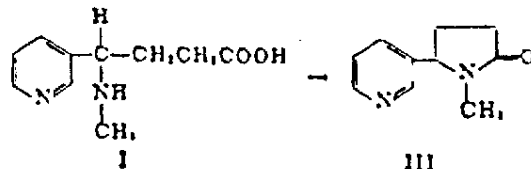
The lactam (IV) was hydrolyzed in hot barium hydroxide solution to yield the desired methylamino acid, with absolute optical configuration corresponding to (-)-nicotine.



The synthetic methylamino acid was investigated by Owen and Larson (1958), who found on the basis of the cyanogen-bromide reaction that about 86% of an administered dose was excreted unchanged by an anesthetized dog. Under similar conditions the radioactivity of nicotine-C<sup>14</sup> (randomly labeled) was excreted to the extent of 93% of the administered dose (Bennett, Tedeschi and Larson, 1954).

In anesthetized dogs under similar conditions, with 10 mg./kg. (-)-nicotine administered slowly intravenously over an 8-hour period and the urine collected from an indwelling catheter during administration and a subsequent 10-hour period, McKennis, Turnbull and Bowman (1957, 1958) obtained evidence for the presence of the suggested metabolite  $\gamma$ -(3-pyridyl)- $\gamma$ -methylaminobutyric acid. Samples of acidified urine were placed on Dowex 50 (H<sup>+</sup>). The pyridine compounds were subsequently removed from the resin with dilute ammonia. The ammoniacal eluate, which on paper chromatograms was shown to contain Koenig-positive components corresponding to  $\gamma$ -(3-pyridyl)- $\gamma$ -methylaminobutyric acid, cotinine, nicotine, and other metabolites, was subjected to treatment on Dowex 1 (OH<sup>-</sup>). This cation-exchange resin retained the suspected  $\gamma$ -(3-pyridyl)- $\gamma$ -methylamino acid. The methylamino acid was then removed with acetic acid and cyclized to cotinine. The latter was identified in the form of its monoperate, m.p. 104-106°C.

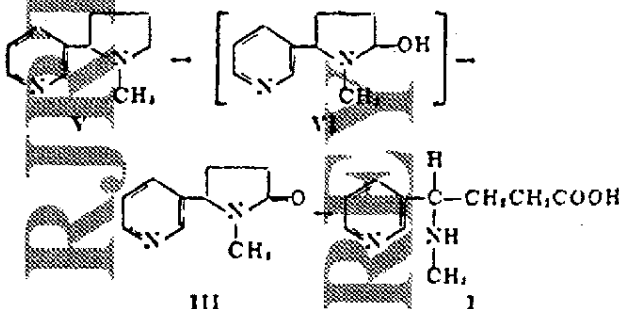
During the course of these studies, it was observed that older samples of urine contained more cotinine than did newer samples (McKennis, Turnbull and Bowman, 1958). In the older samples, the content of  $\gamma$ -(3-pyridyl)- $\gamma$ -methylaminobutyric acid was correspondingly reduced. Studies *in vivo* showed that the spontaneous lactamization



proceeded readily at body pH and temperature. Thus,  $\gamma$ -(3-pyridyl)- $\gamma$ -methylaminobutyric acid can serve *in vivo* as a precursor of cotinine.

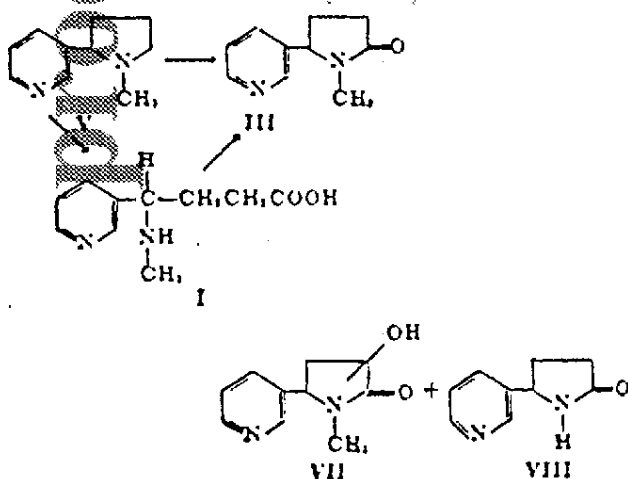
In addition to the foregoing pathway to cotinine, involving the intermediate formation of  $\gamma$ -(3-pyridyl)- $\gamma$ -methylaminobutyric acid, other and more direct pathways may exist.

Frankenburg and Vaitiekunas (J. Am. Chem. Soc. 79: 149, 1957) noted the oxidation of nicotine to cotinine on addition of hydrogen peroxide, or merely upon storage in a bottle. This suggests the possibility that cotinine may arise *in vivo* under the influence of peroxide-catalase (Bowman, Turnbull and McKennis, 1959). Evidence for formation without the intermediate methylamino acid is found also in the work of Hucker and Gillette (1959) and of Hucker, Gillette and Brodie (1959). These investigators, using a purified and fortified liver preparation, noted the oxidation of nicotine (V) to cotinine under conditions not especially conducive to lactamization of the methylamino acid, and suggested the following sequence:



The intermediate alkylol (VI) represents the cyclic form of  $\gamma$ -(3-pyridyl)- $\gamma$ -methylaminobutylaldehyde, which Frankenburg, Gotscho and Vaitiekunas (J. Am. Chem. Soc. 79: 149, 1957) considered as a possible intermediate in the metabolism of nicotine. This hypothetical intermediate may occupy a key position in the metabolism of nicotine, since it is on formal grounds an intermediate which would upon subsequent oxidation yield  $\gamma$ -(3-pyridyl)- $\gamma$ -methylaminobutyric acid. No studies *in vivo* or *in vitro* with enzymatic systems have afforded convincing evidence for the conversion of cotinine to  $\gamma$ -(3-pyridyl)- $\gamma$ -methylaminobutyric acid. Studies of the stability of cotinine *in vitro* indicate the necessity for a yet-unknown amidase reaction, if significant hydrolysis of cotinine is to be carried out under physiological conditions (McKennis, Bowman and Turnbull, 1955).

Following administration of cotinine or nicotine, the dog excretes in the urine desmethylnicotine (VIII) and hydroxycotinine (VII). The following metabolic sequence is therefore indicated:



Hydroxycotinine and desmethylnicotine have similar Rf values. Following acetylation, however, desmethylnicotine readily separates from the mixture. Acetylated hydroxycotinine has been obtained in the form of its picric acid salt, m.p. 166.5–170°C.,  $C_{14}H_{17}O_6N_2$  (McKennis *et al.*, 1959). The elementary analysis of these salts is in close agreement with the theoretical values. The strong positive Koenig reactions of the compound point to the probability that the hydroxyl group occupies a position on the pyrrolidone ring. Exact assignment of the position must await synthetic and degradative studies.

Desmethylnicotine from the metabolism of (-)-nicotine or cotinine is laevorotatory. By reduction of the compound with lithium aluminum hydride in tetrahydrofuran (Wada *et al.*, unpublished results), (-)-normicotine (partially racemized in the reduction) is formed. Since (-)-normicotine has the same absolute optical configuration as nicotine, the biotransformation of nicotine to desmethylnicotine proceeds with retention of the absolute optical configuration of nicotine. Similarly, cotinine and  $\gamma$ -(3-pyridyl)- $\gamma$ -methylaminobutyric acid metabolically formed in dogs from (-)-nicotine have the same absolute optical configuration as the parent compound. (+)-(3-Pyridyl)- $\gamma$ -methylaminobutyric acid is cyclized spontaneously or upon heating to (-)-cotinine (McKennis, Turnbull and Bowman, 1958). The latter, upon reduction with lithium aluminum hydride, yields (-)-nicotine.

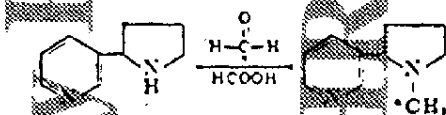
Since the smoking of tobacco leads to the ingestion of nicotine, normicotine (albeit in minute amounts), as well as other pyridine compounds, many investigators have examined the urine of smokers for excretion products (see section on urinary excretion of nicotine in smokers, above). Generally speaking, the urinary excretion of nicotine appears to amount to approximately 10% of the dose. The experiments showing that storage of nicotine and its metabolites in the rat, mouse, and dog is negligible or small (Ganz, Kelsey and Geiling, 1951; Geiling, 1951; Bennett, Tedeschi and Larson, 1954) would suggest that the same would hold for man. The metabolism of nicotine in man has had only limited investigation.

Bowman, Turnbull and McKennis (1959) studied the metabolism of nicotine in a human subject who received orally 30 mg. of (-)-nicotine for 2 consecutive days (in hourly daytime doses of 3 mg. each). An examination of the chloroform-soluble Koenig-positive metabolites by chromatographic methods revealed a striking similarity between the urines of the nicotine-treated human subject, smokers, and the nicotine-treated dog. Although the evidence suggests metabolism of nicotine to cotinine, hydroxycotinine, and desmethylnicotine, only cotinine has been obtained in sufficient quantity for positive chemical identification. This identification was effected by comparison of the mono- and dipicrate salts prepared from authentic cotinine and cotinine isolated from the urine. Following oral ingestion of nicotine, one human subject excreted 10% of the administered dose as cotinine in the urine. A calculation of the percentage conversion by smokers was not made, since the smoking habits of the individuals contributing urine were not available for the study.

Studies on the composition of smoke have indicated the presence of cotinine in tobacco-smoke. The excretion of cotinine by smokers represents, therefore, both exogenous and endogenous material. That the contribution of the former to the total excretion is small, follows from the data of Quin (J. Org. Chem. 24: 914, 1959), which indicates that cotinine is present to the extent of only 0.057 mg. per cigarette in the mainstream smoke. McKennis and Bowman (unpublished results) have shown the human can readily metabolize cotinine, and have demonstrated one or more chloroform-soluble metabo-

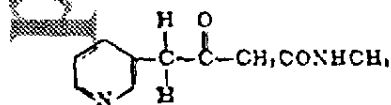
lites in the urines of 2 non-smoking humans following oral administration of the compound. The subjects in these studies received 300 mg. of cotinine daily in single oral doses, and reported a complete absence of the nausea and vomiting which so often follows administration of nicotine. The preliminary data indicate strongly that there may be several metabolites of cotinine in the urine which have not yet been found in the urine of animals or humans following administration of nicotine. One suggestion has been that these differences reflect possible inhibitory effects of nicotine on the further metabolism of its degradation products. The effect of diet, directly on metabolism, or indirectly in possible influences on reabsorption of parent compounds or metabolites from the kidney or bladder, remains also to be considered.

In addition to the foregoing problems which, for any early solution, require the employment of considerable experimental finesse, much is required to elucidate the basic mechanisms which are responsible for the many metabolic transformations of nicotine which have been thus far uncovered. In the metabolism of nicotine to desmethylcotinine, many possible routes are apparent. Following the discovery of this demethylation reaction, McKennis *et al.* (unpublished results) prepared (-)-nicotine- $C^{14}$ -methyl by treating (-)-nicotine with formaldehyde- $C^{14}$  and formic acid:



The labeled nicotine was injected intraperitoneally into rats. Carbon dioxide in the expired air contained approximately 10% of the  $C^{14}$ -methyl group administered, and the urea carbon contained approximately 0.2%. Gant, Kelsey and Geiling (1951) in mice, and Bennett, Tedeschi and Larson (1954) in dogs, had been unable to detect  $C^{14}$  in the expired air; but the opportunity for this in the dose used was diluted through their use of  $C^{14}$ -randomly-labeled nicotine. This, and isolation of desmethylcotinine following administration of nicotine to dogs, clearly establish demethylation, but do not establish whether oxidative or transmethylation processes are involved.

Recently, McKennis, Bowman and Turnbull (1960) have isolated, following the administration of nicotine to dogs, a urinary metabolite with empirical formula  $C_{10}H_{11}NO_2$ . This metabolite yields methylamine and a keto acid upon acidic or basic hydrolysis. It contains one carbonyl group which reacts with hydroxylamine to form an oxime,  $C_{10}H_{11}NO_2$ . Since the product on reduction under Wolf-Kishner conditions affords  $\gamma$ -(3-pyridyl) butyric acid, the position of the side-chain is indicated. The keto acid, which is stable to acidic hydrolysis, differs from the known  $\gamma$ -(3-pyridyl)- $\gamma$ -oxobutyric acid. In consequence, the structure is tentatively given as  $\gamma$ -(3-pyridyl)- $\alpha$ -N-methylbutyramide:



This interesting metabolite could conceivably provide, through possible enzymatic hydrolysis, a source of methylamine as well as pyridyl acetic acid and acetic acid.

Another viewpoint on the path of metabolism of nicotine arose from the findings that liver slices metabolized nicotine [3-(1-methyl-2-pyrryl)pyridine] much more rapidly than nicotine; that the enzyme system involved has much the same

characteristics as that for nicotine; and that the metabolism of nicotine is completely inhibited in the presence of nicotine, but that of nicotine is not affected by the presence of nicotine (Werle and Koebke, 1949). This led to the view that nicotine may be an intermediate product in nicotine metabolism. Pursuing this further, Werle, Koebke and Meyer (1950) examined the urine of dogs, rats, and guinea pigs receiving nicotine for the presence of nicotine by means of the diazo reaction. Distillates from control urines gave a positive diazo reaction, but following nicotine administration, the intensity was increased by an amount calculated to be equal to about 9% of the administered nicotine. In addition, the individual organs of guinea pigs and rats gave intensified diazo reactions for nicotine following intracardiac or intraperitoneal injection of nicotine. From this, the conclusion was reached that nicotine may be a product of nicotine metabolism. Colorimetric, chromatographic, and ultraviolet spectroscopic studies by Takeuchi (1955a, b) were interpreted by him as indicating the presence of nicotine in rabbit-liver extract incubated with nicotine. However, when nicotine was administered to dogs by Larson *et al.* (1950), the urine did not contain an end-product yielding a red color when reacted with cyanogen bromide. Arguing that this product appears to be a product of cleavage of the pyrrolidine ring of the nicotine molecule, and that if nicotine is an intermediate in nicotine metabolism, its formation would precede the ring cleavage, these authors felt that the postulate that nicotine may be an intermediate product in nicotine metabolism was not likely to be true, at least in the dog.

Werle and Meyer (1950) claimed the production of methylamine using liver slices incubated with nicotine, but, later, Schievelbein and Werle (1957) were unable to detect formation of methylamine in the detoxication of nicotine by rabbit-liver slices. Also, following administration of  $C^{14}$ -randomly-labeled nicotine to the dog, Owen and Larson (1955) were unable to detect any radioactivity in a volatile fraction from urine that should have contained any excreted methylamine. These observations lessen the likelihood that 4-(3-pyridyl)-4-keto-butyric acid, a product of metabolism of nicotine by a species of soil bacteria (Wade and Yamasaki, 1953), may be a metabolite of nicotine in mammals. Further indication of this was obtained by Turnbull, Bowman and McKennis (1955), who, in chromatographic studies on the urine of rabbits receiving this acid, found no König-reaction-positive materials corresponding to those obtained from the urine of nicotine-treated dogs. One metabolite of the keto acid, 4-(3-pyridyl)-4-hydroxybutyric acid, was isolated from the rabbit urine and chemically identified.

Nicotine oxide was felt to have been eliminated as a product of nicotine metabolism, since a reduction reaction did not convert the products of nicotine metabolism back to nicotine (Werle, Koebke and Meyer, 1950).

In studies on detoxication of nicotine by liver slices with recovery of the unchanged nicotine by steam distillation, Werle, Koebke and Meyer (1950) noted that the time-extinction curve for the cyanogen-bromide reaction product with this distillate showed a slight but regular deviation from the course of the curve with pure nicotine; also, upon intravenous injection of this distillate, the form of the blood-pressure curve was different from that following injection of pure nicotine, in that a fall in blood pressure followed the rise in pressure produced by nicotine. From the steam-distillation residue, a chloroform- (not ether-) soluble material could be extracted which gave a positive CNBr-aniline reaction; the material was not extractable at acid or neutral pH, and hence was a

base. Assuming the sensitivity of the reaction to be the same as that for nicotine, the material would account for about 30% of the detoxified nicotine. By treating the steam-distillation residue with zinc and acetic acid, there resulted a steam-volatile product which reacted with cyanogen bromide and aniline; it had no pressor effect on the dog, and hence was not nicotine; but its time-extinction curve indicated it was closely related to nicotine.

Werne and Meyer (1950) incubated 400 gamma of several nicotine derivatives with 800 mg. of liver slices, and reported that 42% of anabasine, 34-41% of anatabine, 18-32% of metanicoline, and 18-25% of normicotine were detoxified in a 2-hour period.

From skin and supporting tissue, which are rich in organic sulphur radicals, Jena (Deut. med. Wochr. 53: 240, 1927) prepared a fraction which he called Detoxin. Keeser (1927) found that intravenous injection of Detoxin into pigeons and rabbits inhibited the toxic effects of nicotine, and he suggested that a chemical process was involved in which the sulphydryl groups in Detoxin played an important role. The question of whether loss of SH groups in the ferment system might reduce the detoxication capacity by liver homogenates was investigated by Werle, Schievelbein and Spieth (1956), with negative results. It may be noted that these latter authors have given a general review of what was known prior to 1956 concerning the detoxication of nicotine by the animal organism.

Wada, Kisaki and Saito (1959) isolated and identified nicotinic acid, oxynicotine, nicotyrine, rotinine, and myosmine from the oxidation products of nicotine produced by aeration at 30°C. These results indicate that non-enzymatic reactions may account, to some extent, for transformations of nicotine.

#### Rate of Detoxication

Using the term detoxication in the broad sense of freeing the body of the toxic effects of nicotine by any and all means, the following findings have been made:

Many investigators have noted that nicotine is quite rapidly detoxified by the animal body in that, given fractionally over a period of hours, several times the single lethal dose can be administered without fatal consequences (dog: Dobrzanski, 1926; cat: Eddy and Hitcher, 1925; Straub and Amann, 1940; Travell, Bodansky and Gold, 1940; mouse: Heubner and Papierkowski, 1938; Haag, Larson and Finnegan, 1945; rabbit: Weatherby, 1939). Larson, Finnegan and Haag (1949) studied the relative rate of detoxication of nicotine by several species by comparing the L.D.<sub>50</sub> values, as determined by instantaneous intravenous injection of the total dose under Dial anesthesia, with the lethal dose when given over an 8-hour period by continuous intravenous infusion. L.D.<sub>50</sub> values were: dog, 5 mg./kg.; cat, 2.0; rabbit, 9.4; and mouse, 7.1. Lethal doses over the 8-hour period were: dog, 15 mg./kg.; cat, 22; rabbit, 40; mouse, 40. These results, while comparative, do not represent the maximum rate of detoxication of nicotine by these animals, since the body concentration of nicotine was built up gradually during the infusion. The results do indicate that nicotine is quite rapidly detoxified by the animal body, and that considerable species variation exists in this regard.

Species variation in the rate of detoxication of normicotine has also been shown to occur. Larson and Haag (1943) obtained data indicating that the rabbit may dispose of normicotine at a rate comparable to that found by Weatherby (1939) for nicotine in this species. However, the mouse detoxified nicotine much more rapidly than normicotine (Larson, Haag and Finnegan, 1945). In a more quantitative study in the dog,

Hucker and Larson (1955) found that not only was normicotine metabolized more slowly in this species than was nicotine, but that rate of metabolism appeared to be independent of dosage within the range studied.

Following the injection of nicotine labeled with C<sup>14</sup>, the mouse eliminated 50% of the administered radioactivity in the urine in 6 hours, and the rat eliminated about 40% in 3 hours, about 85% in 6 hours, and all or almost all in 16 hours (Ganz, Kelsey and Geiling, 1951; Geiling, 1951). Similarly, following intravenous infusion over an 8-hour period in the dog of 1 or 10 mg./kg. doses of nicotine containing C<sup>14</sup>-randomly-labeled molecules, approximately 85% of the radioactivity appeared in the urine within 36 hours (Bennett, Tedeschi and Larson, 1954). Twenty-four hours after injection of 5 mg./kg. nicotine into rats, no nicotine could be found in liver, lung, kidney, spleen, intestine, brain, or skeletal muscle steam-distilled in the presence of sodium chloride and magnesium oxide (Werle and Uschold, 1948). Thus, it appears that neither nicotine nor its products of metabolism are retained to any great extent in the body.

Comparable data for man are not available. However, Wolff, Hawkins and Giles (1949) studied subjects smoking 20 cigarettes in 7 hours, and their results indicated quite rapid detoxication of nicotine by man. Control blood, drawn 8-10 hours after smoking, contained nicotine or nicotine-like substances in amounts equal to 0.02-0.35 mg. per liter, and at the end of the smoking period, this was elevated by 0 to 0.13 mg. per liter. Assuming that about 60 mg. of nicotine were absorbed from smoking the 20 cigarettes, the authors estimated that 80-95% was metabolized during the smoking period. The analytical method for nicotine used in these studies was a spectrophotometric one.

The metabolism of nicotine and its detoxication (in the broad sense) have been reviewed by Larson (1952).

#### Metabolism by Microorganisms

Bucherer and Enders (1941-42) succeeded in isolating 3 strains of nicotine-catabolizing bacteria. When incubated at 37°C. for several weeks in a nutrient solution containing nicotine, potassium acid phosphate, magnesium sulphate, and ferrous sulphate, plus fertile earth and sewage sediment, a species of green bacteria catabolized 95% of the added nicotine in 6-8 weeks, a species of brown bacteria catabolized 24.7% in 5 weeks and 100% in 6-8 weeks, and a bacillus X catabolized 100% in 3 weeks. Later, Bucherer (1942-43) described the isolation in pure culture of several species of bacteria which would decompose nicotine; the culture medium used contained nicotine as the only nitrogenous and carbon-containing constituent.

Wenusch (1942c) noted that 0.1% nicotine phosphate solutions to which tobacco seeds had been added and permitted to stand at room temperature for 3 days lost their nicotine content, as judged by silicotungstic-acid precipitation of a steam-distillate or ether extraction of the liquor, both of which methods failed to recover nicotine. The nicotine was not taken up and stored in the seeds, for only trace amounts could be found in these. Of the two possible explanations, that the nicotine was chemically altered by enzymes present in tobacco seed or formed on soaking the seeds, or that the change was caused by bacteria, the latter assumption was held to be the most likely; and Wenusch adduced evidence that the tobacco seeds merely produced a better nutrient medium for the bacteria. The chemical change undergone by the nicotine molecule was considered to be extensive, since even pyridine precipitates with silicotungstic acid. As a result of this bacterial

action, the solution became violet in color. From such solutions, Wenusch succeeded in isolating a hard yellow material, soluble in dilute alcohol, melting with foaming at 250°C.; but whether or not this was related to the destroyed nicotine was not determined. Subsequently, Wenusch (1942d) isolated a material corresponding to *N*-methyl myosmine.

Using a species of bacteria (probably belonging to *Pseudomonas*) isolated from soil, which was able to utilize nicotine as a carbon as well as a nitrogen source, E. Wada and Yamasaki (1953, 1954) analyzed the culture medium following incubation with nicotine, and postulated that the nicotine degradation followed the path: nicotine  $\rightarrow$  oxynicotine  $\rightarrow$  pseudonornicotine  $\rightarrow$  4-(3-pyridyl)-4-keto-butyric acid  $\rightarrow$  ? Structural formulas are given in Wada and Yamasaki (1953). A substance which may be produced through further decomposition was not extractable with ether from acid or alkaline solution, but was precipitated as the picrate from the acidified solution. This had a melting point of 315°C., and analyzed carbon 27.29, hydrogen 1.34, nitrogen 36.77. In further studies, Wada (1955, 1957) found that bacteria which can utilize the tobacco alkaloids as sole sources of both nitrogen and carbon may be classified into two types, A and B, of which type A are only capable of degrading nicotine, whereas type B decompose nornicotine and anabasine as well as nicotine. The degradation course of nicotine by both types was found to be the

same and was represented as follows: nicotine  $\rightarrow$  pseudo-oxynicotine  $\rightarrow$   $\gamma$ -keto- $\gamma$ -(3-pyridyl) butyric acid  $\rightarrow$  3-succinoyl-6-hydroxypyridine  $\rightarrow$  aliphatic compounds. Nornicotine was degraded by the bacteria, type B, along the following scheme: nornicotine  $\rightarrow$  6-hydroxymyosmine  $\rightarrow$  3-succinoyl-6-hydroxypyridine  $\rightarrow$  aliphatic compounds. Anabasine was degraded by the same bacteria along the pathway: anabasine  $\rightarrow$  1', 6'-dehydro-6-hydroxyanabasine  $\rightarrow$  3-glutaroyl-6-hydroxypyridine  $\rightarrow$  aliphatic compounds. The essential action of the bacteria of both types on the alkaloids consists of dehydrogenations and hydroxylations. The sequence of these steps is the reverse in nicotine oxidation, as compared to the oxidation of nornicotine and anabasine. It was noted that nicotyrine, 3-pyridyl methyl ketone, 3-pyridyl *N*-propyl ketone, and oxynicotine cannot be intermediates of nicotine degradation by the bacteria. Nicotinic acid seemed not to be an intermediate in nicotine degradation, but could serve as the sole source of nitrogen and carbon for both types of bacteria, and was degraded through 6-hydroxy nicotinic acid.

Frankenburg and Vaitekunas (1955a) found that microorganisms derived from the surface of tobacco seeds degraded nicotine *in vitro* in  $5 \times 10^{-4}$  to  $5 \times 10^{-3}$  molar aqueous solutions at pH 7. The stepwise oxidative breakdown of nicotine can proceed along three different pathways, as outlined in Figure 1-2. From solutions of nicotine undergoing this change,

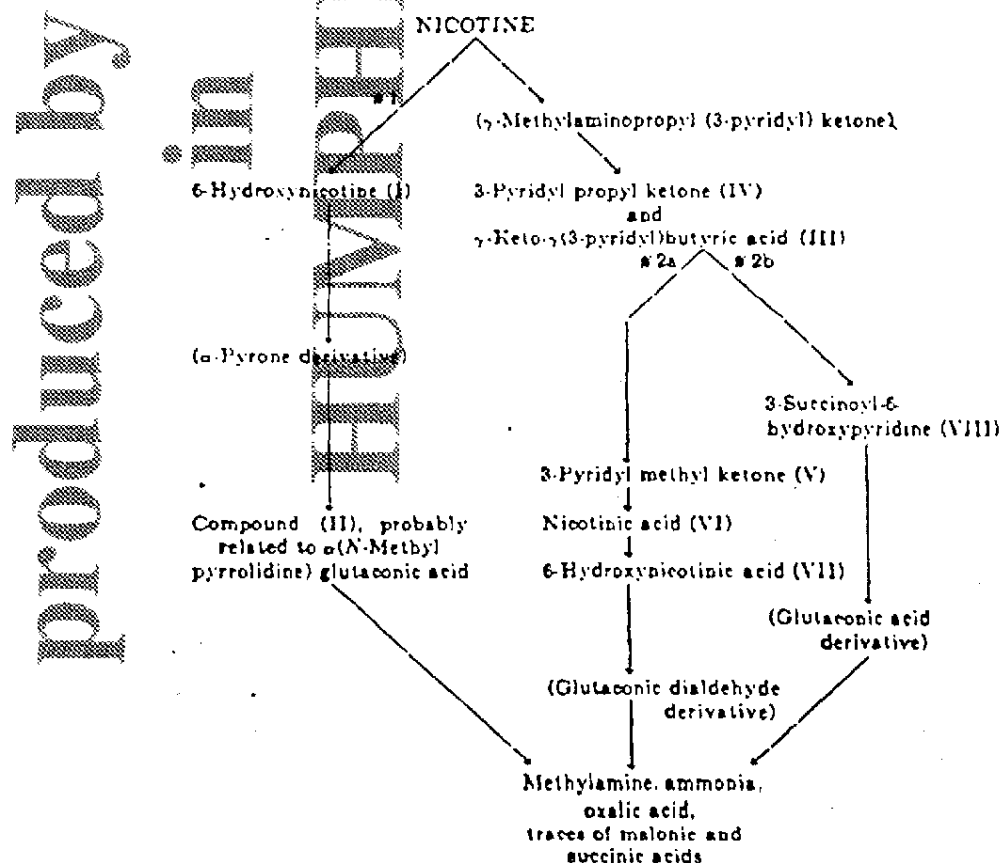


FIG. 1-2. Pathways of oxidative breakdown of nicotine by micro-organisms derived from the surface of tobacco seeds. (From Frankenburg and Vaitekunas, 1955b, courtesy of Archives of Biochemistry and Biophysics.)

Frankenburg and Vaitekunas (1955b) isolated compounds (I) and (II) and the terminally-listed chemicals involved in pathway #1. The other two pathways both start with opening of the pyrrolidine ring, with compound (III) as the first detectable product; (III) is further decomposed by either one of two sequences of reactions, #2a or #2b. The numbered compounds were identified in these pathways (see Figure 1-2).

Hylin (1959) reported that *Acromobacter nicotinophagus* can oxidize nicotine by two distinct pathways. In rapidly dividing cultures, the alkaloid is degraded via 6-hydroxynicotine to aliphatic products. Mature, resting cells convert nicotine to a non-metabolizable product, 6-hydroxy-3-succinoylpyridine. The enzymes which catalyze this transformation are apparently formed adaptively. Interruption of cell division results in adaptation to this second pathway. Other alkaloids studied (normicotine, metanicotine, anabasine, nicotinic acid, and 6-hydroxynicotinic acid) were not metabolized by this organism.

Casida and Rosenfield (1958) isolated from tobacco leaves a bacterial strain which oxidized nicotine to  $\gamma$ -aminobutyric acid. The amino acid was found to accumulate to a limited extent in fermentation broths because the enzyme required for its oxidation is adaptive in nature. Recoveries of 19-26% of the theoretical possible amount of  $\gamma$ -aminobutyric acid were achieved. Data from manometric experiments with resting cells indicated that old cells were able to oxidize nicotine completely and in a linear fashion to  $\gamma$ -aminobutyric acid. Young cells carried out an initial oxidation requiring approximately 5 micromoles oxygen per micromole nicotine oxidized; this was

followed by slower pH-sensitive oxidation steps. It was postulated that the bacterium attacks first the pyridine portion of the nicotine molecule. Glutamic acid would be an intermediate, and would be immediately decarboxylated to give  $\gamma$ -aminobutyric acid.

Crude cell-free extracts prepared from a soil bacterium (designated as strain P-34, a gram-negative rod) capable of growing at the expense of nicotine as the sole source of carbon and nitrogen, were found to degrade nicotine with the consumption of 3 micromoles of oxygen per micromole of nicotine when supplemented with methylene blue; no carbon dioxide was formed up to this level of oxidation (Hochstein and Rittenberg, 1959a, b). With crude extracts, nicotine oxidation proceeded through a series of sharp changes of rate occurring after the uptake of 0.5, or multiples of 0.5 micromoles of oxygen per micromole of nicotine. Chromatographic evidence and ultraviolet absorption data indicated that each point of change of rate coincided with the temporary accumulation of intermediates in the oxidation sequence, and that none of the serially accumulated intermediates are oxidized until their precursors are exhausted. The nature of the first oxidative product, and its failure to give a Koenig reaction, suggest a primary attack at the pyridine moiety of nicotine to yield a pyridone substituted at an  $\alpha$ -carbon of the pyridine ring (1959a). Further studies indicated that the first oxidative product of nicotine was (1)-6-hydroxynicotine, on the basis of its correspondence in properties, including infrared spectrum with synthetic 6-hydroxynicotine (1959b).

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## Addiction, dependence and habitual substance use

David M. Warburton

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law (Title 17 U.S. Code),

For over six months now, I have been haunted by the word addiction. It started when someone asked me at a party, 'You are a psychologist. You do work on smoking. Do you think that smoking is addictive?' I said 'No, not in the sense that addiction is usually used', and said something about smoking not being in the same sort of activity as heroin taking.

I didn't think much more about the conversation until I read in News and Notes of the *British Medical Journal* of 16 June 1984 that:

In the United States smokers with lung cancer continue their efforts to sue the tobacco companies; in its May/June newsletter the Association for Non-smokers' Rights reports that Rose Cipolloni's lawyers are claiming that the companies ... manufactured addictive cigarettes which made health warnings meaningless.

Did this mean that the word addiction could be applied to cigarette smoking after all and that I had been mistaken in my answer?

Later, I noticed a newspaper headline which read 'The Dangers of Addiction' (*Guardian*, 14 August 1984). I started to read it eagerly and discovered that it was an article on the widespread use of computers by children to solve mathematical problems to the detriment of their arithmetic ability. If we assume that this headline was not one of the famed *Guardian* misprints, then what did this sort of addiction mean? I was very puzzled.

A few months after this on the PM programme of BBC Radio 4, there was a discussion on soap operas and the interviewer asked: 'Do people become addicted to soap operas?' The person's answer was 'Of course, they do.' There was no hesitation by the interviewee. By now, I was confused about the use of words like 'addict', 'addicted', and 'addiction' and so I headed for the major dictionaries for help.

### Addiction

In common usage in the United States, addiction is taken to mean habitual behaviour. For example, *Webster's New International Dictionary* (3rd ed.) defines addiction as 'the compulsive uncontrolled use of habit forming drugs' and the addict as 'one who habitually uses and has an uncontrollable craving for an addictive drug'. In this definition, an addictive compound is seemingly only one that can generate uncontrollable craving. In contrast, the *Concise Oxford Dictionary* defines an addict as 'a person addicted to a habit, esp. one dependent on a

(specified) drug'. In the Webster definition, there is no mention of dependence, while the British English definition has no mention of uncontrollable craving.

Obviously, there is not a simple consensus in the English speaking world. In desperation, I looked up the derivation of the words addict, addicted and addiction. The *Shorter Oxford Dictionary* gives the etymology of addict as: from the Latin word, *addictus*—assigned by decree, made over, the past participle of *addicere*, to appoint, allot. Addict in Roman Law meant to deliver over formally by judicial sentence to, and from that, figuratively, to devote or apply habitually to a practice. As examples, it cites:

1. The day he addicts ... to study.
2. He cannot addict his mind to ... profitable business.
3. To addict themselves to Sack.
4. To addict themselves to vice.

From addict has come the past participle 'addicted' used in the sense of devoted to.

Maria: ... and he will smile upon her, which will now be so unsuitable to her disposition, being addicted to a melancholy as she is, that it cannot but turn him into a notable contempt. *Twelfth Night*, II, v. 211.

... but Mr Salteena was not very addicted to prayers so he marched up to bed. Ashford.

We be virgins, and addicted to virginity. Greene.

The noun addiction has come from Roman Law referring to 'a formal giving over by sentence of court; hence, a dedication to a master' and so devotion. However, addiction, as well as meaning devotion, can also mean a bent or an inclination.

Herald: It is Othello's pleasure, our noble and valiant general, that, upon certain tidings now arrived, importing the mere perdition of the Turkish fleet, every man put himself into triumph: some to dance, some to make bonfires, each man to what sport and revels his addiction leads him. *Othello*. II. ii. 6.

Thus we can see that addiction in ordinary usage can refer to work and business, sport and revels, melancholy and study, Sack and vice, prayers and virginity. Certainly, it is a very broad concept which has been applied to a wide variety of behavioural phenomena.

Besides the everyday use of the word addiction, it is reasonable to ask if there is a scientific definition of 'addiction'. A search of the literature reveals many possible medical definitions. One set of these includes physical dependence as an essential characteristic. For example, the National Clearinghouse

for Drug Abuse Information in the 1970s equated addiction with physical dependence on a drug and this is the most common, older definition of addiction. For example:

Addiction occurs only when opiates are used to alleviate withdrawal distress, after this distress has been properly understood or interpreted, that is to say, after it has been represented to the individual in terms of linguistic symbols and cultural habits which have grown up around the opiate habit. If the individual fails to conceive of his distress as withdrawal distress brought about by the absence of opiates he cannot become addicted . . . Lindesmith (1947).

This 'addiction' definition by Lindesmith (1947) requires that there is physical dependence, but also it specifies that there is some recognition by the individual of the connection between use and withdrawal symptoms. Thus, without this awareness, the term addiction cannot be used.

Some definitions, however, have not made physical dependence or withdrawal symptoms a requirement at all. For example, Chapman (1962) proposed:

Drug addiction is the repetitive and compulsive use of some natural or synthetic substance to the detriment of self or society.

In the United States Narcotic Rehabilitation Act of 1966, as quoted in Rappolt (1972), an addict is any individual who habitually uses any narcotic drug so as to endanger the public morals, health, safety or welfare, or who is addicted to the use of such narcotic drugs as to have lost the power of self-control with reference to addiction.

In these terms, an addict is either a person who uses drugs and so endangers moral, health or safety problems, or he may only be a user with no control over their use, or both. Physical dependence or withdrawal symptoms is not a necessary symptom at all in this sort of definition.

Many definitions have not made physical dependence or withdrawal symptoms a crucial requirement but do include it as one of the several possible characteristics, like loss of self-control and harm. The definition of Maurer & Vogel (1962) is one example of this sort of definition.

Drug addiction may be defined as a state in which a person has lost the power of self-control with reference to a drug and abuses the drug to such an extent that the person or society is harmed . . . In addition one or more of the following related but distinct phenomena are always present: (a) tolerance; (b) physical dependence with resulting abstinence illness when the drug is withheld; (c) habituation or emotional dependence.

In these latter definitions, an essential characteristic of addiction is loss of self-control over use and the extent of the harm for the user or society and not physical dependence. However, the statements about 'power of self-control' and 'harm' are so vague as to be almost meaningless.

A rather different definition of addiction was proposed by the World Health Organization Expert Committee on Addiction-Producing Drugs in 1950 and revised in 1957. Their 1957 definition stated:

Drug addiction is a state of periodic or chronic intoxication produced by the repeated consumption of a drug (natural or synthetic). Its characteristics include: (i) an overpowering desire or need (compulsion) to continue taking the drug and to obtain it by any means; (ii) a tendency to increase the dose; (iii) a psychic (psychological) and generally a physical dependence on the effects of the drug; and (iv) detrimental effect on the individual and on society.

It is clear that the WHO definition departs significantly from those of Lindesmith, Maurer & Vogel, and Chapman. In the WHO definition, the essential aspects are an overpowering desire, a tendency to increase the dose and a detrimental effect on both the individual and on society. Physical dependence is not a requirement for addiction, only a possibility. Tolerance is not essential and only a 'tendency' is required. Clear specification of statements such as 'overpowering desire' and 'tendency to increase the dose' were not made. Similarly, 'detrimental effects' is such a value-laden concept that agreement on its nature would be impossible. Indeed, it is doubtful whether any of these terms can easily be specified.

In addition, it is obvious that the condition, 'psychic dependence', if it existed, would be difficult to determine. This has now been recognized by the WHO and the 16th WHO report of the Expert Committee on Drug Dependence (1969) stated:

Evidence concerning the presence and degree of psychic dependence is drawn mainly from case histories, subjective statements and general observation.

In view of these flaws in the definition, it is not surprising that the WHO Expert Committee on Addiction-Producing Drugs (1964) concluded:

The definition of addiction gained some acceptance, but confusion in the use of the terms addiction and habituation and misuse of the former continued. Further, the list of drugs abused increased in number and diversity. These difficulties have become increasingly apparent and various attempts have been made to find a term that could be applied to drug abuse generally. The component in common appears to be dependence, whether psychic or physical or both. Hence, use of the term 'drug dependence', with a modifying phrase linking it to a particular drug type in order to differentiate one class of drugs from another, has been given most careful consideration. The Expert Committee recommends substitution of the term 'drug dependence' for the terms 'drug addiction' and 'drug habituation'.

This recommendation was endorsed by the WHO Scientific Group later in that year (WHO Scientific Group, 1964). The term addiction was abandoned and the WHO Expert Committee on Addiction-Producing Drugs was renamed the WHO Expert Committee on Drug Dependence.



In spite of this rejection of the term. The National Institute on Drug Abuse produced their *Technical Review on Cigarette Smoking as an Addiction* in 1979, in which a NIDA committee gave a definition which was devised specifically to include smoking. Unfortunately, it is susceptible to many of the criticisms of addiction that have been mentioned before. Their wording of the definition was:

An addicting substance is one that has: (1) pharmacological properties leading to compulsive use; (2) a capability of producing organ and/or behavioral toxicity; and (3) a use pattern associated with adverse social consequences. In addition, this term is generally applied when the ingestion of such substances is viewed by a large segment of the society as undesirable.

The definitions that have been cited imply that 'addiction' is drug use that results in dependence, tolerance and the need to increase the dose, and compulsive use resulting from uncontrollable craving in various combinations. However, Stepney (1981) has shown convincingly that certain non-drug habits, such as slimming, athletics, hobbies and gambling, reveal many of these sorts of features as well. For example, he points out that the tolerance is not unique to drug use.

For the confirmed athlete no speed is fast enough, for the stamp collector no specimen rare enough, for the anorexic no weight is low enough, for the businessman no bank balance large enough.

From this description, it is clear that there is no agreement about the definition of the term 'addiction' in the medical and psychological literature; it has often been defined to fit the substances that the investigators have been interested in. The broad definitions include many sorts of habits. Some definitions of addiction imply physical dependence and some do not. Some involve tolerance and the need to increase the dose and some do not. They sometimes specify compulsive use with uncontrollable craving and sometimes they do not. The meaning of the word 'addiction' is thus confusing not only to ordinary people but even to the scientific community. Indeed, as Reginald Smart of the Addiction Research Foundation in Toronto, who listed some definitions of addiction that were mentioned above, concluded: 'In summary, there is no real agreement about concepts such as addiction and abuse' (Smart, 1974; p. 31). If there is no consensus on the definition of addiction, how can the word be used as a health warning on cigarette packs or on advertisements? As psychologists, we should abandon the use of the term which has so many vague connotations. It is significant that the American Psychiatric Association (1980) in their *Diagnostic and Statistical Manual of Mental Disorders* do not use the term 'addiction' for any substance use disorder and instead they use the concept of dependence, e.g. tobacco dependence. The question is whether the concept of dependence is a better concept than addiction.

## Dependence

The WHO definition of drug dependence (WHO, 1969) which was used to replace addiction was:

A state, psychic and sometimes also physical, resulting from the interaction between a living organism and a drug, characterised by behavioral and other responses that always include a compulsion to take the drug on a continuous or periodic basis in order to experience its psychic effects, and sometimes to avoid the discomfort of its absence. Tolerance may or may not be present.

Unfortunately, this definition is as unsatisfactory as that of addiction because it still involves vague concepts such as 'overpowering desire', 'psychic dependence', 'deprivation symptoms' and 'tendency to increase dose'. It is so broad as to be almost meaningless. The only sort of substance use that could be excluded might be a chemical from which the user derived no benefit after continued use. Certainly coffee drinking is included, and many forms of chronic medication, like hormone therapy and pain killers, would fit as well. The definition would even encompass eating as a form of chemical dependence.

The American Psychiatric Association (1980) in their *Diagnostic and Statistical Manual of Mental Disorders* include problem use as one of their diagnostic criteria. Thus, for tobacco dependence the definition is:

- A. Continuous use of tobacco for a least one month.
- B. At least one of the following:
  - (1) serious attempts to stop or significantly reduce the amount of tobacco use on a permanent basis have been unsuccessful;
  - (2) attempts to stop smoking have led to tobacco withdrawal;
  - (3) the individual continues to use tobacco despite a serious physical disorder (e.g. respiratory or cardiovascular disease) that he or she knows is exacerbated by tobacco use.

The American Psychiatric Association note specifically with respect to impairment that:

Since tobacco use rarely causes any identifiable state of intoxication as does alcohol, there is no impairment in social or occupational functioning as an immediate and direct consequence of tobacco use.

The main criteria for which they recognize dependence is (a) when the person is ill and this illness is aggravated by continuing to use tobacco, and (b) that when people, who wish to give up for whatever reason, cannot do it. This definition does not involve vague concepts such as 'overpowering desire', 'psychic dependence', 'deprivation symptoms' and 'tendency to increase dose'. It merely argues for a diagnosis of dependence when there are problems associated with substance use.

This definition recognizes that dependence is a condition that exists in many forms and many degrees, and it is not only with extreme use that problems can occur. Dependence has many aspects to it, rather than any one key diagnostic feature. We

are all 'dependent' for our ordinary happiness, gratification, emotional well-being and general quality of life on a whole range of people and objects. We are in this sense clearly dependent on things like our family and friends, our job, our motor car, our favourite armchair and the television set. We experience 'withdrawal symptoms' if we are deprived of them. It is consistent with this view that some people find that their quality of life is improved by the use of chemical substances, like a glass of beer, a cup of coffee and a cigarette and some degree of substance dependence on alcohol, coffee and cigarettes. If the word is used in this way, dependence must be considered a normal condition. Among normal drinkers and smokers there will be a wide range of sensed need for alcohol, coffee and cigarettes, in terms both of smoking and drinking occasions and the amount consumed. Thus everyone who smokes or drinks is at least in some minor degree dependent on cigarettes, coffee and alcohol. They suffer 'deprivation' symptoms if they did not have their evening glass of sherry or their after-meal cigarette and coffee. The crucial issue is not the dependence but the consequences that may result from habitual substance use.

#### Habitual substance use

The terms 'addiction' and 'dependence' are what Christie & Bruun (1969) referred to as 'fat words'; they bring under single headings types of behaviour that are extremely disparate. In all definitions of addiction and dependence that have been discussed, the one common factor is substance use. Recently, this kind of approach has been followed by the National Research Council of the United States. They sponsored a series of meetings and reports on habitual substance use with the aim of finding common biological, psychological and social processes, underlying the habitual use of substances, including alcohol, food, tobacco, and psychoactive drugs. The results of this research are published in *Commonalities in Substance Abuse and Habitual Behavior* (Levinson et al., 1983).

Among the most important considerations of any studies of substance use is the sort of use that will result in problems for the individual and society which is called substance abuse. Unfortunately, the articles in the *Commonalities in Substance Abuse and Habitual Behavior* volume do not consider the extent of use that will result in problems. In order to reduce the likelihood of 'problems', it will be necessary to understand safe substance use. As Orford (1985) has pointed out in his book, *Excessive Appetites*, many substances are used by the majority of people as a pleasurable and moderate form of indulgence without problems.

#### Conclusion

The terms 'addiction' and 'dependence' are vague terms about which there is considerable disagree-

ment. Their definitions have been used to encompass under single headings types of behaviour that are extremely disparate. It is important for future research, public health policy and education that psychologists should not use terms like addiction and dependence if we cannot say what they mean precisely. It is the recommendation of this paper that psychologists would do better to consider substance use and not substance 'addiction' or substance 'dependence'. If we know more about safe substance use, then optimization of use should be possible and we will be in a better position to consider the issues of substance abuse in terms of the individual and society.

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January 20, 1998

Donald Kennard  
Attorney at Law  
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1200 Main Street  
Kansas City, MO 64105

RE: Engle et al v R. J. Reynolds et al

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December 20, 1997

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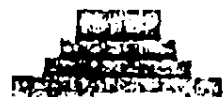
  
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July 18, 1997

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Engler v Brown &amp; Williamson et al

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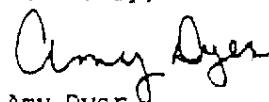
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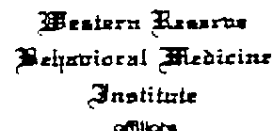
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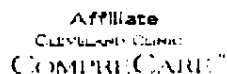
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June 17, 1998

VIA FEDERAL EXPRESS

A. James Giannini, M.D.  
5144 Yellow Creek  
Poland, OH 44514

Re: B&W/Endo

Dear Dr. Giannini:

Enclosed is a list along with copies, of various documents that we understand you already have reviewed. Please call me once you have reviewed these materials.

Best Regards,

Sincerely,

*Joseph H. Hunt*  
Joseph H. Hunt

HJH:mym  
Enclosures

cc: Richard A. Schneider (w/out enclosures)

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- (a) Nicotine inhibits food intake, either in acute administration to "fresh" rats or in chronic administration ("tolerant" rats). The threshold value for "fresh" rats was found to be the same as for the antidiuretic effect and the action on ACTH release (0.5 mg/kg).

The action of nicotine in the appetite test was not modified by reserpine administration; the investigation of the effect of adrenaline in this test was in agreement with this fact, and led us to the conclusion that nicotine does not act on food intake via the release of nor-adrenaline. Nicotine acted normally on rats lesioned in the satiety centre.

- (b) Nicotine stimulates very rapidly the mobilization of lipidic depots: as early as 10 minutes after the administration of the drug, the lipids are mobilized and free fatty acids appear in blood in greater amounts. The threshold value of nicotine activity was found once more to be the same as in the other tests.

- (c) When one waits until six hours after the administration of nicotine, the free fatty acids present in blood are diminished, and their disappearance is proportional to the dose of nicotine administered.

This fact shows that nicotine enhances the destruction of the products of lipid mobilization.

We were therefore in a position to show that nicotine bears a three-fold activity against obesity, viz.:-

- (i) Anti-appetite effect
- (ii) Mobilising effect
- (iii) Stimulation of FFA degradation.

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4. A Possible Activity of Nicotine on Other Hypothalamic Functions  
(Thyrotropic regulation and gonadotropic regulation)

Such an activity of nicotine was investigated: it was shown that it did not influence at all these hypothalamic functions.

In addition to the above investigations, the main data were discussed, and the necessity to pursue a more thorough investigation of the influence of nicotine in the "stress" reaction was emphasized.

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C O N T E N T S

SUMMARY

I. INTRODUCTION

II. ACTION OF NICOTINE ON THE MECHANISM OF DIURESIS  
Antidiuretic Effect

III. ACTION OF NICOTINE ON THE "STRESS" MECHANISM  
Corticotropin-releasing Effect

IV. ACTION OF NICOTINE ON BODY-WEIGHT REGULATION  
Anorexic Effect  
Effect on Lipid Metabolism

V. POSSIBLE ACTIONS ON OTHER HYPOTHALAMO-  
PITUITARY FUNCTIONS

Thyrotropic Function  
Gonadotropic Function

VI. DISCUSSION AND GENERAL CONCLUSIONS



## I. INTRODUCTION

It is an everyday experience to each smoker that smoking a cigarette helps mastering the numerous stressful stimuli of modern life.

This effect is possibly one of the most powerful reasons which make one smoke.

How does nicotine exert this action? The normal defence mechanism against stressful agents is a nearly immediate release of those hormones synthesized by the adrenal cortex which act upon the cell metabolisms: they are called "corticosteroids" and play the cardinal role in the defence of the organism against stress. Their release from the gland is mediated through a very complicated system involving the stimulation of hypothalamus and pituitary functions.

It is well known that the hypothalamus acts upon the pituitary function through chemical mediators of polypeptidic nature.

One of these hypothalamic mediators is called vasopressin and bears anti-diuretic activities. It is also a stimulator of the pituitary corticotropic function, i.e. the stimulation of corticosteroid release by the adrenal cortex.

It has been shown by Burn et al.<sup>(1)(2)</sup> (1945-1951) and by Bisset and Walker<sup>(3)</sup> (1957) that nicotine caused a profound antidiuretic effect in man and in rat.

- (1) BURN, J., TRUELOVE, C.H. and BURN, I., 1945, The antidiuretic action of nicotine, Brit. Med. J. 1, 403 (1945).
- (2) BURN, J., Antidiuretic effect of nicotine and its implication Brit. Med. J. 2, 199 (1951).
- (3) BISSET, G.W. et al., The effect of nicotine, etc., on the secretion of the antidiuretic and oxytocic hormones of the rat, Brit. J. Pharmacol. 12, (4), 461 (1957).

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As a working hypothesis we assumed the idea that nicotine could help to master the stressful stimuli by way of enhancing (or facilitating) the normal defence mechanism.

If this were true, it would be easier to understand another very important effect of smoking: it is well known that stopping to smoke has an immediate effect on body weight. As body weight is regulated by the hypothalamo-pituitary system, our working hypothesis could be enlarged in order to assume the idea of an interference of nicotine in the hypothalamic regulation of body weight as well as in the defence against stress.

The present work was undertaken in order to verify this working hypothesis through investigations of the following points:-

1. Verification and more thorough study of the action of nicotine on the mechanism of diuresis.

2. Possible interference of nicotine in the "stress" mechanism.

3. Mode of action of nicotine on body weight.

This effect could be obtained by one or all of the following activities:-

(a) Inhibition of appetite

(b) Stimulation of the mobilization of lipid deposits

(c) Stimulation of the degradation of mobilized lipids.

4. Whether or not nicotine interferes in other hypothalamo-pituitary functions, such as thyrotropic or gonatropic regulation.

IMAGE

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## II. ACTION OF NICOTINE ON THE MECHANISM OF DIURESIS (ANTIDIURETIC EFFECT)

### A. Development of Tolerance to Nicotine in Rats

We define the terms:-

- |                  |   |
|------------------|---|
| "fresh rats"     | as rats having never received nicotine prior to the experiment;   |
| "tolerant rats"  | as rats having developed, after a prolonged daily treatment, tolerance to nicotine such as they can bear without fatal reaction doses that would be lethal to fresh rats;   |
| "resistant rats" | as rats that do not develop tolerance while submitted to a prolonged daily treatment by nicotine. This state of resistance may last some weeks, but all the chronically treated rats become "tolerant" after a sufficient time (3 to 4 months). |

The investigation of the various effects of nicotine was made on these three groups of rat.

IMAGE

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B. Investigation of the Antidiuretic Activity of Nicotine  
on Fresh Rats, Tolerant Rats and Resistant Rats

1. DEMONSTRATION OF AN ANTIDIURETIC EFFECT OF NICOTINE

The antidiuretic action of nicotine is well known to many smokers. It is due to the release of the antidiuretic hormone (also called vasopressine, as it bears a vasopressive activity at the same time), which is synthesized by the hypothalamus.

This fact is the basis of our working hypothesis.

We first reproduced this action qualitatively on 10 tolerant and 5 resistant rats, with an additional group of 5 controls having never received nicotine (fresh rats).

All these rats received 2 mg/kg of nicotine subcutaneously at time 0.

The amount of urine released by each rat was measured every hour for 8 hours after the injections, and once after 23 hours.

The results are gathered in Fig. 1. It can be seen that the urine release is completely stopped from the 2nd to the 8th hour after the injection of nicotine to fresh rats (controls) as well as to resistant rats. Tolerant rats, which are less sensitive to nicotine, show only a diminishing production of urine, but no stoppage.

Very similar curves were obtained three times, each time with a different group of rats.

EXACT IMAGE

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2. LOG-DOSE RESPONSE TO NICOTINE IN THE ANTIDIURETIC TEST

Fig. 2 shows the curve obtained in the quantitative antidiuretic test, with doses from 0.5 to 1.0 mg/kg.

The quantitation of the test was made after Burn<sup>(4)</sup> in calculating the time (in minutes) in which 50 % of the cumulative amount of urine was excreted. This length of time is of course augmented by an antidiuretic substance, as can be shown by the response to vasopressine, which is the antidiuretic hormone secreted by the hypothalamus. An approximate threshold of nicotine activity should be fixed at 0.5 mg/kg.

C. Possible Action of Nicotine via the Release of Nor-Adrenaline

Professor J.H. Burn<sup>(5)</sup> has shown that the action of nicotine on the peripheral circulation is due to the release of nor-adrenaline, that follows upon the administration of nicotine.

It was necessary to check whether this hypothesis could be verified on the antidiuretic activity of nicotine.

As it is known that nor-adrenaline is completely discharged from the tissues by the action of reserpine in 16 hours at the doses of 0.5 to 1.0 mg/kg<sup>(6)</sup>, we tried to reproduce the activity of nicotine in the antidiuretic test after the administration of reserpine 16 hours prior to the test.

(4) BURN, J.H., 1931. Quart. J. Pharm. Pharmacol. 4, 517.

(5) BURN, J.H., The action of nicotine on the peripheral circulation. Annals N.Y. Acad. Sc. 90 (1), 81-84.

(6) BERTLER, A., Effect of reserpine on the storage of catecholamines in brain and other tissues. Acta Physiol. Scand. 51 (1), 75-83, 1961.

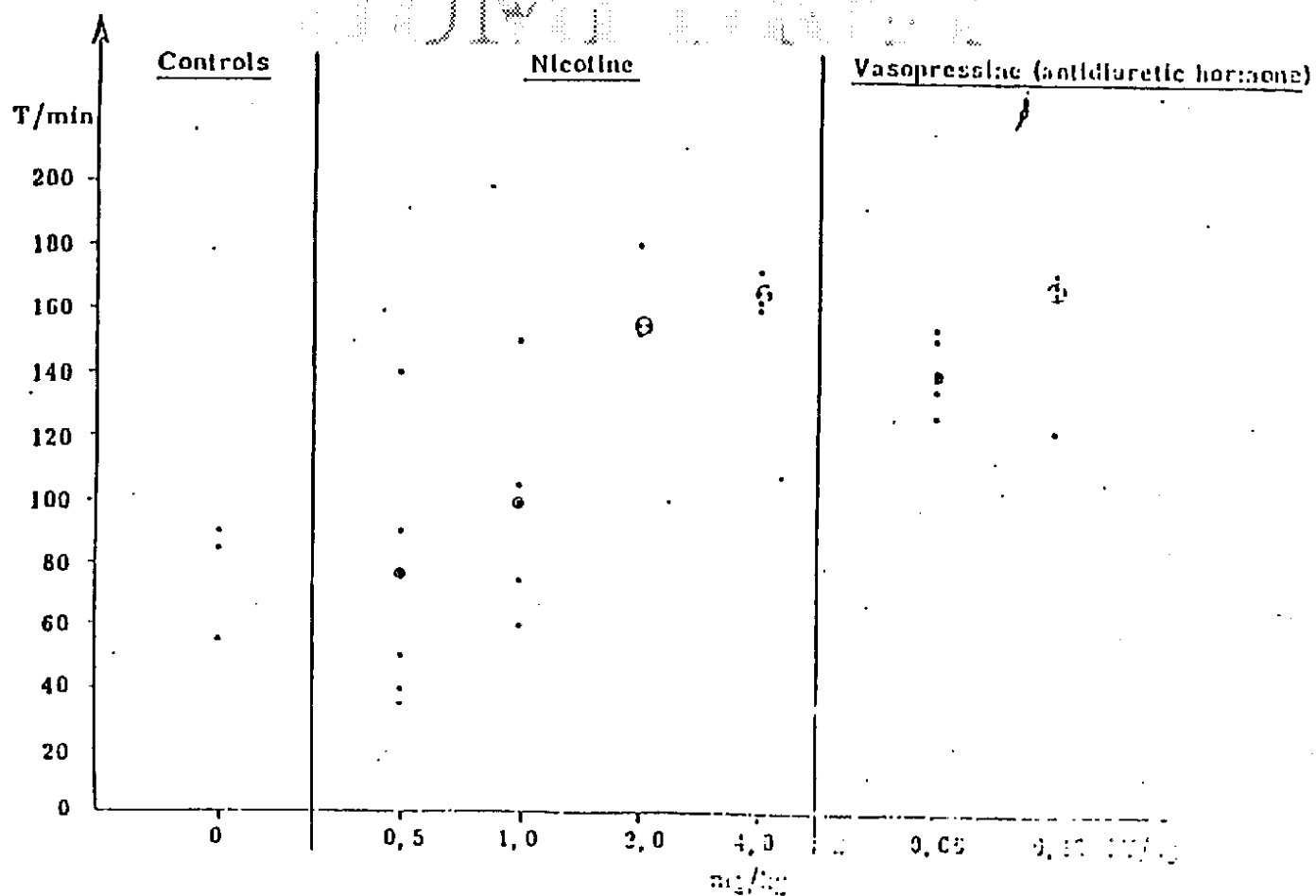
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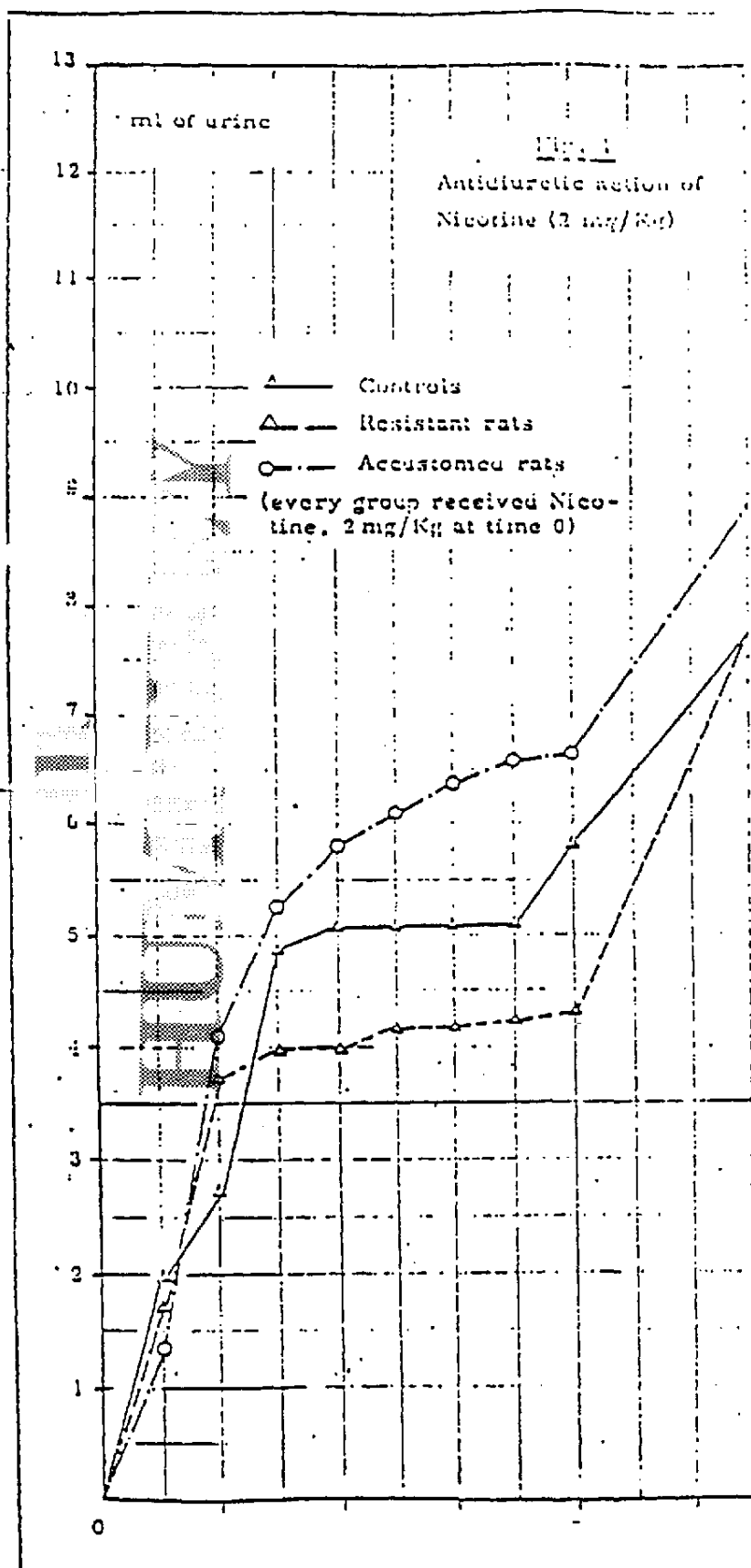


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Fig. 2 Log-dose response to nicotine in the antidiuretic test



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tion

The results are gathered in Fig. 3. It is clear that nicotine has the same action with this pre-treatment than without it, and therefore that the elimination of the stores of adrenaline in tissues prior to the administration of nicotine does not affect its antidiuretic activities. These data do not support the idea of nicotine acting via the release of nor-adrenaline in this test.

D. Investigation of the Influence of Hypothalamus Lesioning  
on the Antidiuretic Effect of Nicotine

Twenty male rats (body weight 188 to 234 g) were operated: 13 were lesioned by electro-coagulation in the hypothalamus region that stimulates the antidiuretic activity (anterior hypothalamus), 7 were "sham-operated", i.e. were operated without electro-coagulation, and served as controls.

The stereotaxic apparatus necessary to perform such operations is a holder for the animal and for the electrode with which the electro-coagulation into the brain is performed. The head of the animal is invariably held exactly in the same position. The co-ordinates of the extremity of the electrode are calculated in order that they correspond to the exact point of the hypothalamus to be lesioned, taking as "point zero" the middle of the axis of the ears of the animal. The measurements are made on the apparatus by way of a three-dimensional micrometer held by it, and to which the electrode is attached.

Among the 13 lesioned animals, 10 survived. They were tested at the same time as the sham-operated animals for diuresis and for the antidiuretic activity of nicotine, from two to three weeks after the operation.

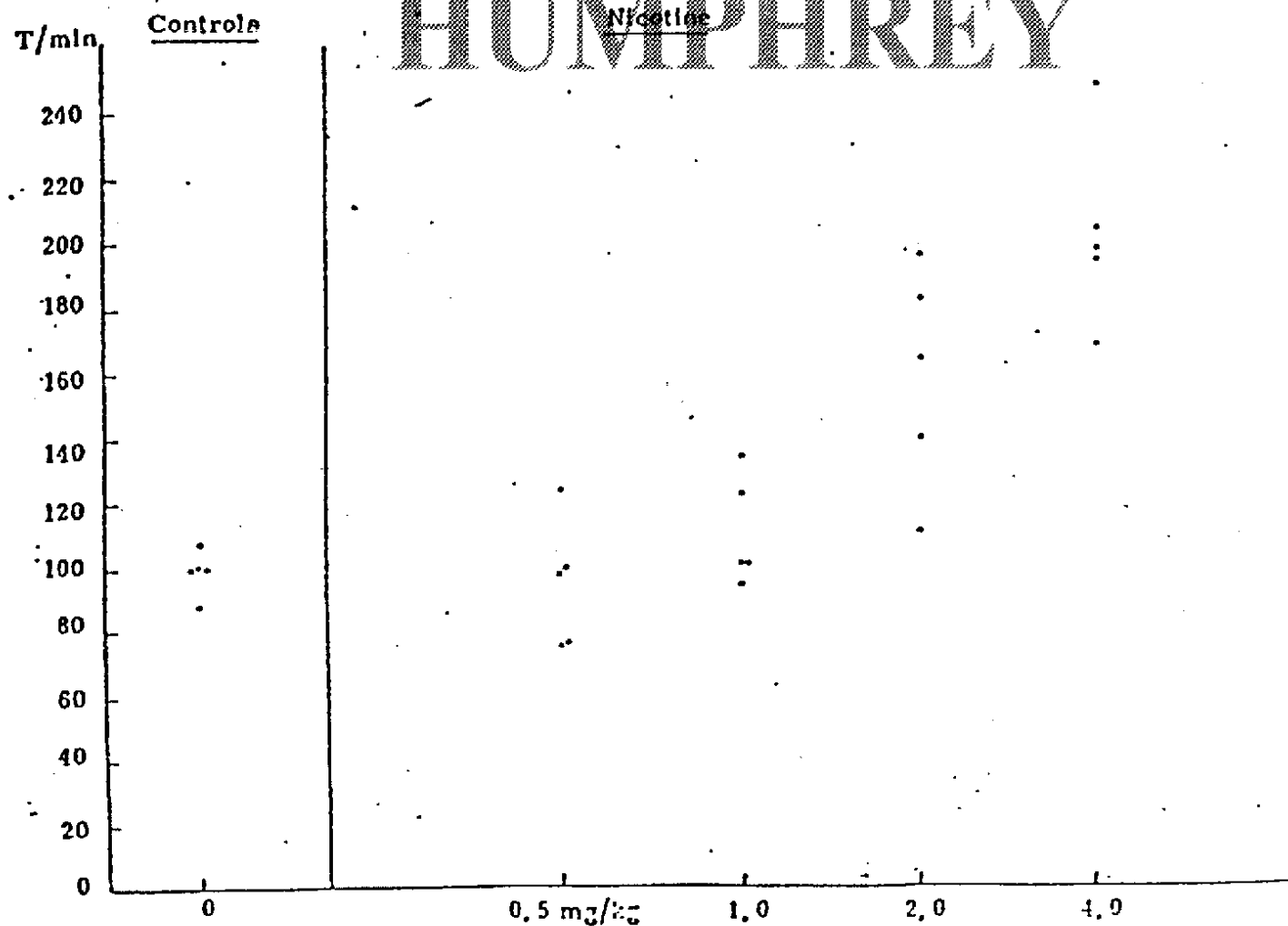
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Fig. 3

Log-dose response to nicotine in the anti-diuretic test, 16 hours after Reserpine(0.5 mg/kg)



The measurements of the antidiuretic activity was made by the usual test of Burn, as described previously. Calculations were done after 270 minutes. The test was performed in two steps, with a one-week interval: the first time, half of the animals (sham-operated and lesioned) received saline, the other half receiving nicotine (4 mg/kg); the second time, the groups were reversed.

Our results are gathered in Fig. 4. This figure shows that nicotine does not seem to stimulate the hypothalamic centre that synthesizes the antidiuretic hormone, as it acts normally after lesioning of this centre.

Nicotine could also act in inhibiting some inactivating mechanism (or centre) of the synthesis of the antidiuretic hormone.

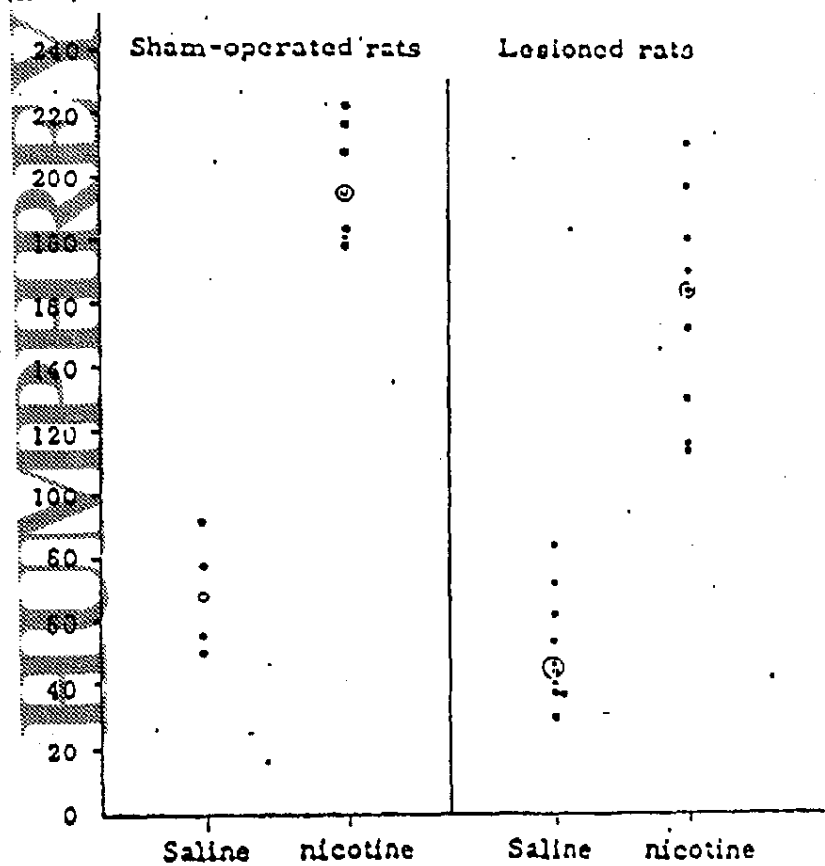
### III. ACTION OF NICOTINE ON THE "STRESS" MECHANISM (CORTICOTROPIN-RELEASING EFFECT)

The demonstration of an influence of nicotine in the normal mechanism of defence of the organism against stressful agents would be of fundamental importance, as is already expressed in our Introduction.

As the normal defence against stress is a release of the pituitary corticotropic hormone (ACTH), the aim of the investigation was to determine whether or not the administration of nicotine increases ACTH release.

Time for 1/2 final  
volume (min)

Fig. 4 Action of nicotine on lesioned rats in the antidiuretic test



The measurement of ACTH release can be made by several tests:-

- (i) Determination of the adrenal ascorbic acid: this substance is involved in the synthesis of the corticosteroids by the adrenals and disappears from the adrenal tissue as the corticosteroids synthesis proceeds. An increased ACTH function is immediately followed by a depletion of the ascorbic acid in the adrenals, lasting some hours. This fact is used as a test of measurement of ACTH function: it is called the "adrenal ascorbic acid depletion" (AAAD) test.
- (ii) Determination of the corticosteroids released by the adrenals. Under the influence of ACTH, the adrenal tissue releases a larger amount of corticosteroids either in blood (in vivo) or in a surviving medium (in vitro).

#### A. Action of Nicotine in the AAAD Test

##### 1. LOG-DOSE RESPONSE

With the AAAD test, the log-dose response to nicotine is very similar to that obtained in the antidiuretic test, and the threshold value can be defined similarly as being about 0.5 mg/kg. as can be seen from the data gathered in Table 1.

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Table 1

Action of Nicotine in the AAD Test

(Adrenal ascorbic acid content in mg/100 g adrenals)

<u>Controls</u> (fresh rats receiving Saline only)	<u>Nicotine-treated rats</u> (fresh rats receiving nicotine in saline)		
	1 mg/kg	2 mg/kg	4 mg/kg
394	293	193	192
328	307	259	212
357	317	269	216
378	341	208	234
392	351	300	237
412	354	337	243
430	359	367	267
456	394	373	269
458	410	380	296
492	422	400	319
505	437	414	340
	454	473	346
Mean: 410.2	369.9	338.8	264.7



## 2. POSSIBLE ACTION VIA THE RELEASE OF NOR-ADRENALINE

In a second step of this investigation we wanted to ascertain whether nicotine in this test acts in releasing nor-adrenaline from the hypothalamus. We therefore reproduced the experiment on "fresh" rats having received reserpine 16 hours prior to the test (see Chapter I, Section C). We obtained the data gathered in Table 2.

Table 2

Action of Nicotine in the AAAD Test  
after Reserpine Treatment

(Adrenals ascorbic acid content in mg/100 g adrenals)

Absolute controls	Reserpine controls	Nicotine (4mg/kg) after reserpine
381	287	205
356	290	241
400	310	245
407	337	203
425	394	328
436		379
Mean: 401	343.6	260

The action of nicotine seems slightly inhibited after reserpine administration: the difference in the means for the reserpine controls and the 4 mg/kg nicotine-treated rats was about 64 mg/100 g adrenals, whereas it was about 145 mg/100 g adrenals when the rats were not treated by reserpine prior to the test.

Nicotine does, however, act on this test after complete nor-adrenaline release.

3. EFFECT OF NICOTINE AFTER CHEMICAL BLOCKADE OF THE  
HYPOTHALAMUS

Briggs and Munson<sup>(7)</sup> have studied the influence of morphine on ACTH release. They have shown that morphine completely blocked ACTH release owing to the administration of histamine, and they have stated that this fact was due to a hypothalamus blockade after administration of morphine. The measurement of ACTH activity was made by the AAAD test.

We wanted to investigate such an effect of morphine prior to the administration of nicotine.

The experiment was thus repeated on animals having received morphine prior to nicotine, exactly in the same way as did Briggs and Munson in the case of histamine instead of nicotine.

The data were the following (Table 3):-

- (7) BRIGGS F.N. and MUNSON P.L., Studies on the mechanism of stimulation of ACTH secretion with the aid of morphine as a blocking agent. *Endocrinology* 57, 205-218 (1955).

Table 3

Action of Nicotine in the AAAD Test  
after Morphine Treatment

Absolute controls	Morphine controls	Nicotine after morphine
261	286	187
296	291	209
305	324	214
324	339	256
330	341	269
334	345	272
350	356	281
371	357	283
379	369	293
388	388	320
404	400	323
409	421	352
Mean: 355	351	271

These data clearly showed that, while morphine given in these conditions did not alter the normal adrenal ascorbic acid content<sup>\*)</sup>, nicotine depleted this content after morphine administration; contrary to histamine, although this depletion was less prominent with morphine than without this preliminary treatment (Δ from nicotine to morphine controls = 80 mg/100 g adrenal).

<sup>\*)</sup> This fact agrees with Briggs and Munson's data.

To summarize the present situation as regards ACTH release by nicotine, we may make the following observations:-

- (i) Nicotine releases ACTH as measured by the AAAD test; this action shows the usual threshold, and is definitely proportional to nicotine dosage.
- (ii) This nicotine activity is slightly inhibited, but not completely abolished, by the following treatments:-
  - nor-adrenaline disappearance from tissues after reserpine
  - hypothalamus blockade by morphine.

It therefore seems that the activity of nicotine on ACTH release - as measured on the AAAD test - which appears quite definite, is probably a very complicated process and needs urgently to be more thoroughly investigated.

Such an activity could very well explain the fact that nicotine helps to master stressfull stimuli, the normal reaction against such stimuli being an enhanced release of ACTH by the pituitary.

Nicotine thus appears to stimulate this normal reaction to stress.

#### B. Effect of Nicotine on the Release of Corticosteroids From the Adrenals

We chose to follow the test of D. de WIED (1961)<sup>(8)</sup> for the measurement of corticotropin-releasing principles. The assay is made

(8) WIED D. de, An assay of corticotropin-releasing principles in hypothalamic lesioned rats. *Acta Endocrinologica* 37, 288-297 (1961).

on "rats with lesions in the median eminence of the hypothalamus, 18 hours after electro-coagulation. In vitro steroidogenesis by adrenal glands is used as a parameter of pituitary-adrenal activity. The blocking effect of the lesion on the release of ACTH in response to stress is measured by the effect of ether anaesthesia on corticoid production in vitro of the left adrenal, whereas steroidogenesis of the right adrenal is used as an index of the corticotropic effect of corticotropin-releasing principles."

The measurement of in vitro steroidogenesis is made following SAFFRAN and SCHALLY technique as described by J. Van der VIES (1957)<sup>(9)</sup> (extraction of the steroids from the medium with methylene chloride, and measurement of the absorption at 240 m $\mu$ ).

#### 1. MEASUREMENT OF IN VITRO STEROIDOGENESIS BEFORE AND AFTER A STRESSFUL STIMULUS

The assay of pure corticosterone dissolved in methylenechloride was fairly reproducible and gave a good curve (difference between optical densities at 240 m $\mu$  and 260 m $\mu$ ; Beckmann D.U. apparatus). 86.5 to 105.8 % of the quantity of corticosterone added to the medium was recuperated.

We then measured the amount of steroids released in a Krebs-Ringer oxygenated medium (Warburg flasks; 37.5°C; gentle shaking for two hours) by adrenals removed from intact adult rats anaesthetized with ether.

(9) VIES, J. van der, Experience with an assay of ACTH based on the steroid output of rat adrenals in vitro.  
Acta Physiol. Pharmacol. Neerland. 5, 361-384 (1957).

The left adrenal was removed within one or two minutes after the rat had been anaesthetized in the animal house; the right gland was taken out 15 minutes after the removal of the left one; the animal woke up in the meantime. Two adrenals of one side were put together into a Warburg flask.

The difference between the amount of steroids released by the right adrenals (surgical stress) and the left ones (rest state) was significant as can be seen from our data (Table 4):

Table 4

Action of a Surgical Stress on Corticosteroids

Release in Vitro

Corticosteroids in  $\mu$ g per 100 mg adrenal

Rat No	Left adrenal	Right adrenal	$\Delta$ % left adrenal
1 + 2	17.4	24.5	+ 40.6
3 + 4	15.6	26.0	+ 66.7
5 + 6	13.6	25.2	+ 85.3
7 + 8	12.7	18.5	+ 45.3
9 + 10	19.7	36.0	+ 83.0
11 + 12	23.9	29.5	+ 23.5
13 + 14	21.5	30.7	+ 43.1
15 + 16	22.8	42.1	+ 85.0

These data confirm that the technique is suitable for measuring in vivo ACTH release, as was stated by the authors.

## 2. ACTION OF NICOTINE

22 rats were used in two groups (12 control rats and 10 rats treated by 4 mg/kg nicotine).

Our schedule was the following :-

- Time 0 : anaesthesia of the rats with Nembutal.  
20 min. : removal of the left adrenal of every rat, followed immediately by injection of saline (control group) or nicotine. The left adrenals of two rats are put together in each Warburg flask.  
35 min. : removal of the right adrenal of every rat; two right adrenals are put together in each Warburg flask.

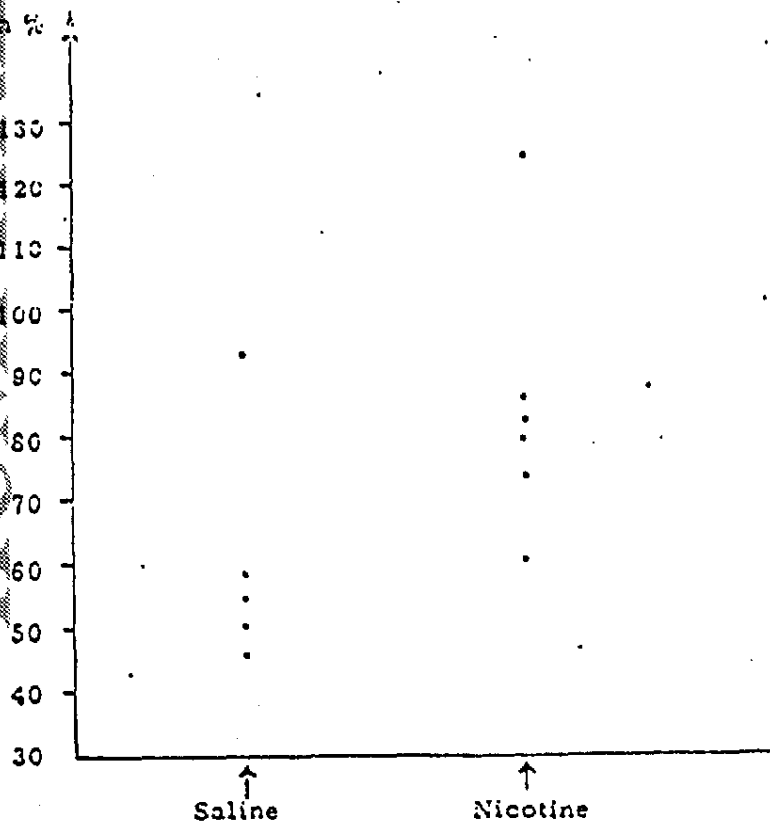
All the flasks are put in a water bath ( $37.5^{\circ}\text{C}$ ) and shaken for 90 minutes. After this time an aliquot of the medium is taken out from every flask, extracted by methylenechloride, and the corticosterone is measured in methylenechloride with the Beckmann D.U. apparatus.

Data are shown in Fig. 5.

Every point represents the difference between the corticosteroids released by 2 right adrenals and the corticosteroids released by the 2 left adrenals of the same rats.

This figure shows a definite increase in this difference when the rats are treated by nicotine. This fact clearly indicates that nicotine enhances the normal reaction to stress, and is in agreement with the results of the AAAD test.

Fig. 5 Action of nicotine on corticosteroids release





#### IV. ACTION OF NICOTINE ON BODY-WEIGHT REGULATION

It is well known that some persons who suddenly stop smoking rapidly gain weight. As smoking does not seem to have any importance on gastric secretions<sup>(10)</sup>, the stopping of it must act in increasing the food intake. It could also act in interfering with the lipid metabolism.

The hypothalamus controls the food intake by way of two antagonistic centres<sup>(11)(12)</sup>. It is possible to make obese hyperphagic rats by lesioning the centre that normally provokes satiety. Hypothalamus is also known to regulate the lipid mobilization from the body depots.

Our working hypothesis was therefore to suppose an enhancing effect of tobacco smoke on the hypothalamic inhibition of appetite, or an inhibiting action on the reverse hypothalamic effect. It seems very likely that this action of tobacco smoke would be due to nicotine itself, either directly or indirectly in releasing adrenaline and nor-adrenaline from tissues<sup>(13)</sup>. It was possible moreover to consider a stimulation of the lipid mobilization following nicotine administration, by way of an enhancement of this hypothalamic function.

(10) WALKER, J. M., Physiological effects of smoking.  
Proc. Nutrition Soc. 12, 157-160, 1953.

(11) HETHERINGTON A. W. and RANSON S. W., Anatom. Rec.  
72, 159, 1940.

(12) BROBECK J. R. et al, Yale J. Biol. Med. 15, 831, 1943.

(13) BURN, J. H., Brit. Ass. for the adv. of Sc., Cardiff, 6th Sept.  
1960.

Consequently, we wanted to answer experimentally the following questions:-

- Is nicotine that factor present in tobacco smoke which inhibits the appetite ?
- If so, does nicotine act by a direct stimulation of the hypothalamic centre that diminishes the food intake, or via the action of adrenaline and nor-adrenaline ?
- Is there an influence of nicotine on the lipid metabolism ?

In order to answer these questions, we made the following investigations:-

- A. Observation of the weight curves of the animals receiving nicotine twice daily, 5 days a week, for the development of tolerance.
- B. Measurement of the food intake in the following groups of rats:-
  1. Fresh rats receiving one injection of nicotine
  2. Tolerant rats during a rest period, as compared with normal controls
  3. Tolerant rats during the injection period, as compared with themselves during the rest period
  4. Normal rats receiving adrenaline and rats pretreated by reserpine
  5. Lesioned rats.
- C. Possible influence of nicotine on the lipid metabolism.

A. Observation of the Weight Curves of the Rats Chronically  
Treated by Nicotine

A typical weight curve is represented in Fig. 6 (rats chronically treated by nicotine for the development of tolerance).

The mean weight increase was calculated separately for males and for females; weighings were made twice a week, on every Monday and Thursday. From these curves we can make the following observations:-

- The weight increase is greater in the control groups than it is in the nicotine-treated groups; the difference is statistically significant after 99 days; however, this difference between control and treated animals is much more significant for males than it is for females (one-tailed probability of error :  $\alpha_{1-t} = 0.43\%$  for males;  $3.67\%$  for females)\*).
- Whenever there is an interruption of the injections, the treated male and female rats tend to gain weight more rapidly than they do during the injection period. This fact is first observed on the curves as occurring every week-end, and it explains the characteristic pattern of the nicotine curves, with alternative periods of rapid weight increase (week-end) followed by weight-loss periods.

Such a pattern is never to be seen on the control curves. Secondly, a similar fact occurred incidentally during an 11-day interruption (Christmas period). This interruption of the injection led to an observable increase of weight in the nicotine group, whereas the controls grew very regularly at the same rate as usual.

\*) All the statistical tests employed in this Report are non-parametrical.

The answer is the percentage of the food intake after the injections against the food intake on the day before the injection calculated for each rat. This test has been used in our laboratory for some months, and we already know that it can respond to the administration of hypothalamic extracts. It is therefore a good test for the measurement of the hypothalamic function in the control of food intake.

### Results

#### 1. FRESH RATS TREATED BY NICOTINE

One group of 32 male rats, habituated to the appetite test, but having never received nicotine before, was given nicotine (2 mg/kg in one subcutaneous injection at 3 a.m.). The test was made in two days, with a one-day interval between the two test-days. On the first test-day half of the rats received nicotine, the other half being given saline; both halves were reversed on the second test-day. The food intake of each rat on the nicotine-day was then compared to that on the saline-day, and the percentages were calculated. The results show a statistically highly significant diminishing activity of nicotine ( $\alpha_{1-1} \approx 0.001 \%$ ). The mean food-intake after nicotine for this experiment is 74.5 % of the mean food-intake before nicotine.

The log-dose response in this test was drawn for the usual doses (1.0 to 4.0 mg/kg). The dose of 0.5 mg/kg still seems to be the approximate threshold value (Fig. 7).

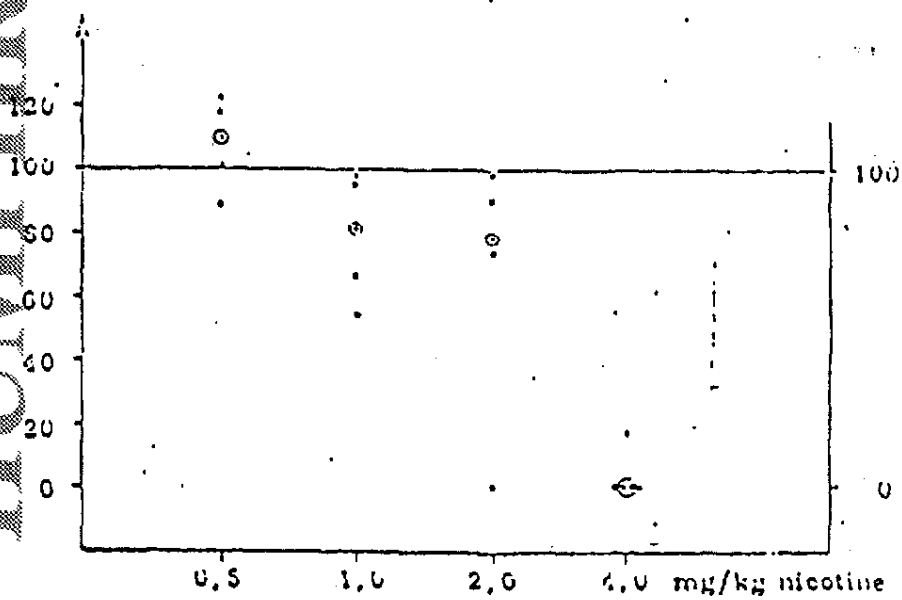
It is therefore absolutely established that nicotine in itself is the factor present in tobacco smoke which inhibits appetite.

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HUMPHIREX

Fig. 7 Log-dose response to nicotine in the appetite test



## 2. TOLERANT RATS DURING A REST PERIOD

Twenty-two tolerant rats were not given nicotine for five days (Saturday and Sunday, as usual, plus Monday, Thursday and Wednesday) and were tested for appetite on the 4th and the 5th day of the rest-period.

The mean food-intake per rat for the 22 rats tested for two days is 1.90 g/hour/100 g rat (range = 1.43 - 2.72). We compared these data with the mean food-intake per rat observed for 40 male rats of the same age and weight as those of the nicotine group, receiving only saline. The mean food-intake of the control rats is 2.46 g/hour/100 g rat (range = 1.43 - 3.30). The difference between these two groups is highly significant (probability of error:  $\alpha_{2-t} = 0.03\%$ ). The mean food-intake of the accustomed rats for this experiment is 77.2 % of that of the control rats.

Tolerant rats therefore eat significantly less food than do fresh rats, even while their organism contains no nicotine at all (5 days after the last injection).

An attempt was made at comparing the food intake for male and for female tolerant rats during the rest period: under these conditions female rats eat significantly ( $\alpha_{1-t} = 3\%$ ) more food than male rats (mean food-intake = 2.05 g/h for females instead of 1.76 g/h for males).

## 3. TOLERANT RATS DURING THE NICOTINE-INJECTION PERIOD

The same 22 tolerant rats as those in 2. were tested for food-intake during the injection period, the amount of food eaten in one hour being measured from 2.30 to 3.30 hours after a nicotine injection of 2 mg/kg.

This test was performed on the week before the interruption period described in 2. The mean food-intake per rat for the 4 days of the test was 2.05 g/hour/rat (range = 1.40 - 2.50). From these results, as compared with those reported on in 2., it seems that there is no difference between the food intake of accustomed rats during the injection period and during a rest-period of 5 days. This fact does not seem to corroborate our findings on the weight curves.

One explanation of this discrepancy could be that nicotine acts against weight increase not only in inhibiting appetite, but also in stimulating metabolism, i.e. in stimulating utilization and destruction of the reserves (fat depots, muscle glycogen, etc.).

#### POSSIBLE EFFECT OF NICOTINE VIA THE RELEASE OF ADRENALINE AND NOR-ADRENALINE

##### (a) Action of Adrenaline and Nor-Adrenaline in the Appetite Test

We assayed the effect of adrenaline in the appetite test in exactly the same way as we did for nicotine, i.e. by injecting the rats at 8 a.m. (100  $\mu$ g) and measuring the food eaten from 10.30 to 11.30 a.m. Under such experimental conditions there was a tendency towards a diminished food intake, but this effect was not statistically significant ( $\alpha_{1-2}$  = 3.1 %).

Nor-adrenaline, in the same dosages and under the same experimental conditions, acted very slightly against food-intake, much less markedly than did nicotine (probability of error,  $\alpha_{1-2}$  = 2.6 %, mean food-intake = 95.6 % of the control period).

We then tried to measure a possibly more rapid anorexic action of nicotine, adrenaline and nor-adrenaline : if nicotine acts by way of releasing the catecholamines, these latter must act more rapidly than does nicotine itself.

The one-hour tests gave the following results : (a) for the probability of error  $\alpha$  with which we may assert a difference between the test-period and the control-period; (b) for the mean food-intake % of the control period in this experiment:-

	$\alpha_{1-t}$	Mean food-intake % of control period
Nicotine	< 1 %	62.7
Adrenaline	$\approx 6.2 \times 10^{-8}$	68.3
Nor-adrenaline	little or no difference at all	103.9

These results show that, whereas nicotine and adrenaline are very active one hour after the injection, nor-adrenaline does not yet inhibit the appetite after this lapse of time. The action of nicotine is a lasting one, for we found a significant increase in appetite after 2.30 hours, and even, in the tolerant rats, 5 days after the last injection.

On the contrary, the action of adrenaline is very fugacious, as it is no more significant after 2.30 hours. Nor-adrenaline bears only a very slight activity after 2.30 hours, and none at all after one hour.

An attempt was made at comparing the activities of the three products, in calculating in each case the proportion of rats in which appetite would be diminished in a large population (P dim.). The results of these calculations are gathered in Table 5.



Table 5  
Proportion of Rats in Which Appetite Would be Diminished  
in a Large Population  
(with a 5 % two-tailed probability of error)

(a) Measurement of food-intake 2.30 hours after the injection			
Nicotine	71 %	< P dim.	< 96.5 %
Adrenaline	46.8 %	< P dim.	< 81.5 %
Nor-adrenaline	50.0 %	< P dim.	< 83.9 %
(b) Measurement of food-intake one hour after the injection			
Nicotine	63.5 %	< P dim.	< 92.8 %
Adrenaline	79.2 %	< P dim.	< 99.2 %
Nor-adrenaline	34.7 %	< P dim.	< 70.9 %

It thus does not seem very likely that the lasting action of nicotine against food-intake and weight increase could be due to the release of the catecholamines. Among them, nor-adrenaline is only very slightly and slowly active against food-intake. Adrenaline is more markedly active, but its action is very fugacious and does not last for more than two hours. On the contrary, nicotine acts as rapidly as adrenaline, and its action lasts much longer than that of adrenaline.

(b) Action of Nicotine After the Release of Nor-adrenaline  
(Reserpine Pre-treatment)

The dose of 0.5 mg/kg of reserpine was administered to 20 rats 16 hours prior to the appetite test. Table 6 groups our results in this experiment.

Table 6

Response to Nicotine in the Appetite Test, 16 Hours After  
Administration of Reserpine

Rat	Food intake (g/100 g body weight) in one hour, 16 hrs after reserpine		Action of nicotine (food intake in % of control period, after a week's rest)
	Controls : saline	Nicotine 2.0 mg/kg (after a week's rest)	
1	0.69	0.22	31.9
2	0.77	1.09	142.0
3	1.64	0	0
4	0.26	0	0
5	2.44	0	0
6	2.10	0	0
7	0.24	0	0
8	1.13	1.36	120.0
9	0.85	0	0
10	0.42	0	0
11	1.86	0.67	35.0
12	0.71	0.40	56.5
13	2.00	0	0
14	1.53	0.73	47.5
15	1.09	0.20	18.3
16	1.50	0	0
17	1.45	0	0
18	1.14	0.57	50.0
19	2.26	0	0
20	2.66	0.89	33.4

In comparing these data with those in Fig. 7 (dose 3.0 mg/kg) it can be seen that there is no diminution of the inhibiting activity of nicotine on food-intake when all the stores of nor-adrenaline are eliminated prior to the administration of nicotine. It does not seem likely, therefore, that nicotine acts on appetite by way of the release of nor-adrenaline. These data agree with our first ones as already mentioned.

#### 5. ANOREXIC EFFECT OF NICOTINE ON LESIONED RATS

Twenty female rats weighing from 182 to 242 g were operated: twelve were effectively lesioned in the hypothalamic centre that provokes satiety, and eight were "sham-operated" in the same region. Eleven of the lesioned rats survived.

The mean weight curves of the entire two groups of rats are shown in Fig. 8. Among the eleven surviving lesioned rats, eight showed a higher weight increase during the first two weeks after the operation than during the pre-operation period. However, this increase in growth did not last more than two weeks, and the weight curves became parallel to each other.

This weight increase was due to a definite enhancement of the food-intake, as can be seen in Fig. 9(a).

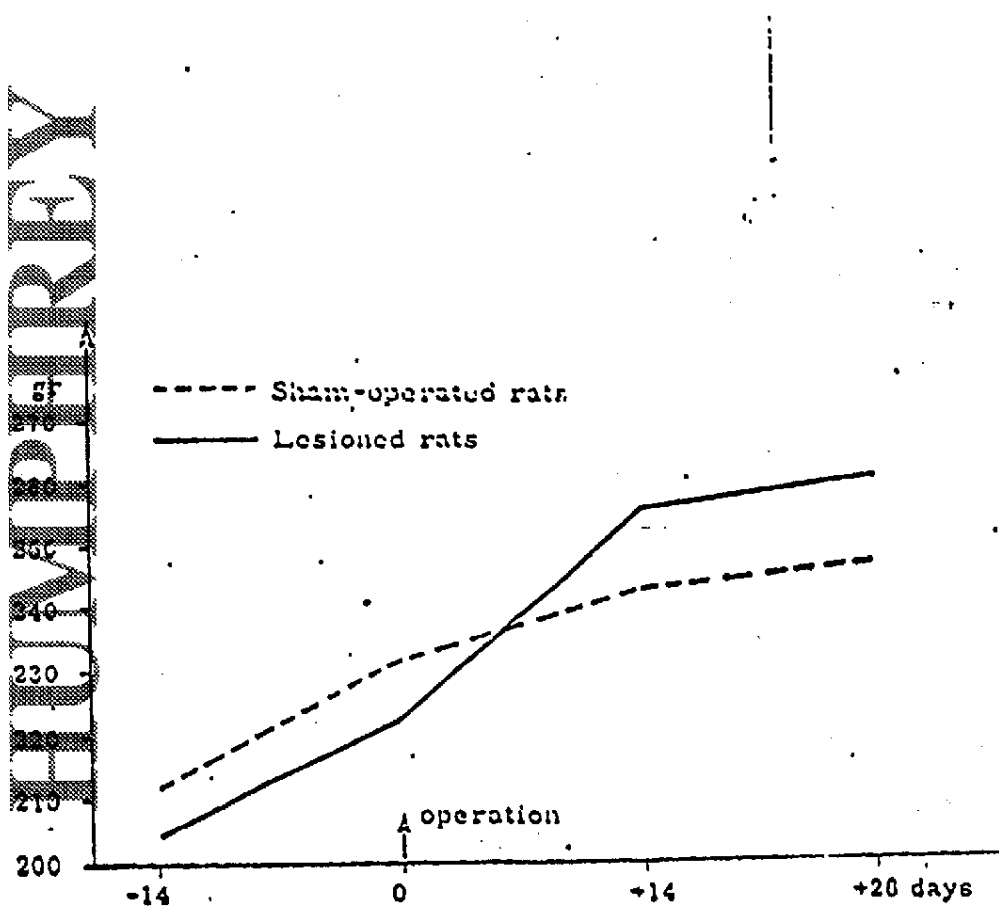
A "crossed test" for the measurement of the effect of nicotine on appetite was made on the whole group of rats, 14 and 21 days after the operation; the data are gathered in Fig. 9(b).

This figure shows that nicotine still acts normally after the lesion.

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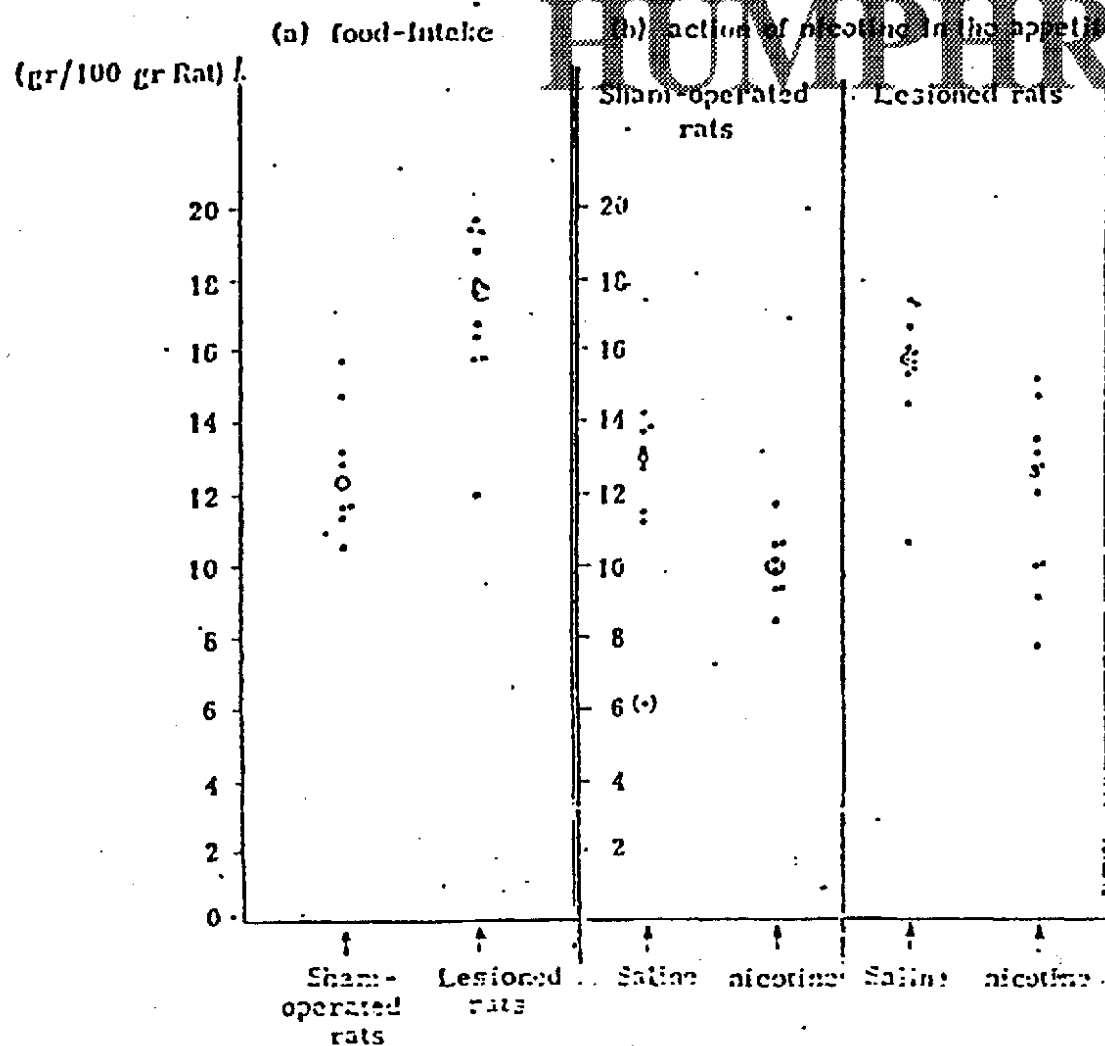
TEMPERATURE

Fig. 3 Curve weights for lesioned and sham-operated rats  
(means of the groups)



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Fig. 9 Lesioned rats ; food-Intake and action of nicotine



It does not seem, therefore, that effective lesioning of the hypothalamic centre which provokes satiety interferes with the anti-appetite activity of nicotine.

This fact could be explained by an inhibiting effect of nicotine on the second known hypothalamic centre regulating the appetite, that centre which activates the food-intake.

#### Action of Nicotine on Lipid Metabolism

Lipid measurements in epididymal fat pad, in liver and in "carcass" were made on a total of 219 chronically treated male and female rats (5 to 25 rats per group). Treatment lasted from two to seven months ( $2 \times 2$  mg/kg/day of nicotine, five days a week). Table 7 groups our results (means and significance), and allows the following observations to be made:-

1. Nicotine acts as a "lipid mobilizer", for it diminishes the amount of fat depots in the epididymal fat pad as well as in the "carcass", whereas fat depots in liver are augmented. This "lipid mobilizing" effect is significant after five months treatment.
2. This fact confirms our idea that nicotine not only is active against appetite but also contributes to diminishing the formation of fat depots.
3. It is well known that some pituitary hormones are "lipid mobilizers" and that a centre located in the hypothalamus itself mobilizes fat depots; it is possible that nicotine acts on this endocrine system.

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Table 7

Chronic Action of Nicotine on Lipid Metabolism (tolerant rats)

Exposure duration of treatment	2 months				4 months				5 months				6 months				7 months			
	♂		♀		♂		♀		♂		♀		♂		♀		♂		♀	
	Controls	Nicotine treated	Controls	Nicotine treated	C	NI	C	NI	C	NI	C	NI	C	NI	C	NI	C	NI	C	NI
Body weight (g)	373	402 $\alpha > 10\%$			583	493 $\alpha > 10\%$			667	461 $\alpha < 5\%$			641	451 $\alpha < 2\%$			558	342 $\alpha < 0.1\%$		
SS %	8.01	6.17 $\alpha > 10\%$																		
%	7.21	7.20 no diff.	6.82	7.02 no diff.							6.03	6.86 $\alpha < 0.1\%$			6.79	6.97 $\alpha < 5\%$			6.03	6.31 $\alpha < 10\%$

\*  $\alpha$  = probability of error for a difference between treated and control animals

† too small number of animals, but tendency to an augmentation

As this action seems extremely important both from the scientific point of view and in the interest of the Tobacco Industry, we tried to learn something more about it.

- (a) The measurement of lipids in carcass was made on 17 chronically treated female rats (2 x 2 mg/kg per day for 60 days) and on 17 controls (same age, same weight) having received saline instead of nicotine for the same period and in the same way (sub-cutaneously).

The results (see Table 8) were extremely interesting and showed a definite inhibition of the accumulation of lipid depots, owing to the chronic administration of nicotine, under our experimental conditions.

Table 8  
Carcass Lipids After Two Months,  
(Chronic Nicotine Administration (mg %))

<u>Controls</u>	<u>Treated</u>
4.74	4.69
8.35	4.82
9.24	5.00
9.26	5.11
9.40	5.42
9.77	5.58
10.23	5.73
10.23	6.07
10.29	6.36
10.54	6.46
11.10	6.68
11.73	7.32
11.90	7.52
12.96	8.21
13.33	8.38
13.88	8.71
14.63	13.11



These data, as compared with the results obtained already with males treated for the same lapse of time of two months (see Table 7), seem to show that the action of nicotine is more pronounced in females than in males. However, we must point out that the number of male controls was small (5 only), so that it is possible that this difference in significance was only due to this fact.

- (b) This action on lipid metabolism could be very interesting if a new way of administering nicotine could be designed. It represents in fact, with the effect on the stress reaction, the only two nicotine effects that could be claimed as "beneficial", and it seems that any publicity around a new gadget could not avoid being based on such a beneficial activity.

To assay the activity of nicotine administered by the alternative ways that will be investigated in the future, we studied a rapid test based on the above-mentioned effect.

The first step of an "anti-obesity" effect is the "mobilization" of the lipid depots and the release from them of free fatty acids (FFA) in the blood.

This step takes place very rapidly, and Kerschbaum et al. <sup>(15)</sup> have shown a 10-minute release of FFA in blood after smoking.

We investigated the "regression curve" of nicotine on fresh rats (i.e. rats having never received nicotine), at two time-intervals after the subcutaneous injection of the usual nicotine doses (1 mg/kg, 2 mg/kg and 4 mg/kg), i.e. after 10 minutes and after 6 hours.

(15) KERSCHBAUM A., DELLET S., DICKSTEIN E.R., and FEINBERG I.J.,  
Effect of Cigarette Smoking and Nicotine on Serum Free Fatty Acids.  
Circulation Research 11, 631-638, 1961.

Fig. 10 groups the results of both experiments.

These graphs show:-

- (i) That nicotine release FFA in 10 minutes, on rats as well as on men, and in amounts directly proportional to the dose of nicotine. This effect is a true "mobilizing" effect.
- (ii) That this mobilizing effect is followed by an enhancing of the disappearance of FFA from blood; that is to say, by a stimulation of the degradation processes of these free fatty acids. This activity is definite after six hours, and is proportional to the dose of nicotine. To our knowledge, it had never been shown until now, and it would deserve a thorough investigation with labelled fatty acids.

Both activities ( i and ii) lead to the very marked decrease in the amount of fat depots in the body that we observed in (2).

Actually, nicotine bears a three-fold activity against obesity:-

- Anti-appetite effect
- Mobilizing effect
- Stimulation of FFA degradation.

It follows that nicotine is a very potent drug - probably the most potent from among those available now - against obesity.

And, now, as the blood is very rapidly mobilized  
into cholesterol and other elements. This is the  
very dangerous phenomenon. And it is the only  
way to be free of it.

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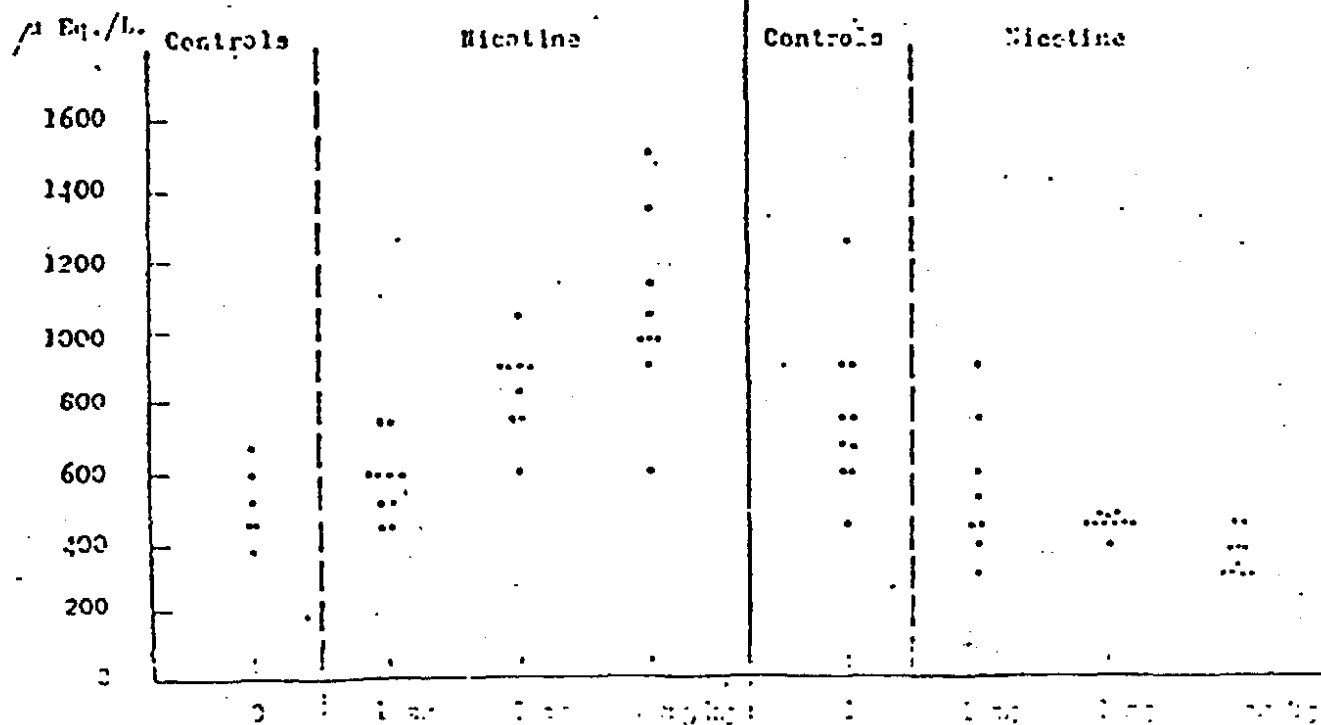
Fig. 10

Nicotine Effects on the Heart Rate of the Rat

HUMPHREY

(a) 10 minutes after nicotine injection  
(animals non fasted)

(b) 6 hours after nicotine injection  
(all animals fasted for 6 hours)



## V. POSSIBLE ACTIONS ON OTHER HYPOTHALAMO-PITUITARY FUNCTIONS

- A. Thyrotropic function (regulation of the thyroid activity)
- B. Gonadotropic function (regulation of the sexual activity).

### A. Investigation of a Possible Thyrotropin-releasing Activity of Nicotine on Fresh Rats

We thought it necessary to enlarge upon our study of the influence of nicotine on hypothalamus activities, and we first chose to investigate a possible effect of nicotine on the release of thyrotropic hormone (TSH - thyreostimulating hormone).

Biological experiments have shown that the anterior hypothalamus plays a part in the control of the thyrotropin release of the pituitary<sup>(16)</sup>.

Work conducted in Japan by SHIBUSAWA and his group<sup>(17)</sup> has demonstrated the existence of a chemical factor contained in hypothalamus extracts that enhances the release of this pituitary hormone. This chemical factor is called "thyrotropin-releasing factor" (TRF).

We therefore tried to determine whether nicotine acts on the hypothalamus in stimulating TRF synthesis.

(16) HARRIS G.W., Neural control of the pituitary gland, Arnold, London, 1955.

(17) SHIBUSAWA K. et al, Endocrinol. Japan 6, 131, 1959.

## 1. PHYSIOLOGICAL TESTS

We used two tests:-

- (a) A qualitative test that makes it possible to follow the function of the thyroid gland when submitted to the action of the hypophyseal thyrotropin, either direct or via the hypothalamic factor.
- (b) A quantitative test that indicates the measurement of thyroid activity at a given time and permits a calculation of the thyrotropin released by the action of any other drug administered.

Both tests are based on the measurement of radio-activity present in the thyroid gland after the administration of  $I^{131}$  to rats.

(a) The qualitative test is based on the fact that, after a period of iodine trapping by the thyroid, lasting some hours, the radio-element is then slowly discharged from the gland, in the form of the thyroid hormones.

It is possible to follow this discharge by radio-activity countings on the neck of the animal, at various intervals of time between about 24 hours and 15 days after the administration of  $I^{131}$ .

The radio-activity of the thyroid measured in this way decreases exponentially.

The administration of the pituitary thyrotropic hormone accelerates the rate of loss of radio-iodine (i. e. the rate of discharge of the thyroid hormones), as was shown first by Harris<sup>(16)</sup>.

The administration of crude hypothalamic extracts reproduces exactly the effect of thyrotropin, as has been shown by Shibusawa<sup>(17)</sup>.

We first verified on our rats the data of Harris and those of Shibusawa, and we found a good thyrotropin-releasing activity in crude hypothalamic extract (Fig. 11).

The test is thus capable of detecting an enhanced synthesis of TRF in the hypothalamus of our experimental rats.

Our attempts at investigating a possible enhancing activity of nicotine and of the catecholamines on the release of thyrotropin, as measured by the rate of discharge of the thyroid hormones, did not show any notable activity of the drugs assayed on the rate of release of thyroid hormones (Fig. 12 a and b).

It seems, therefore, that the drugs assayed do not enhance thyrotropin release by the pituitary.

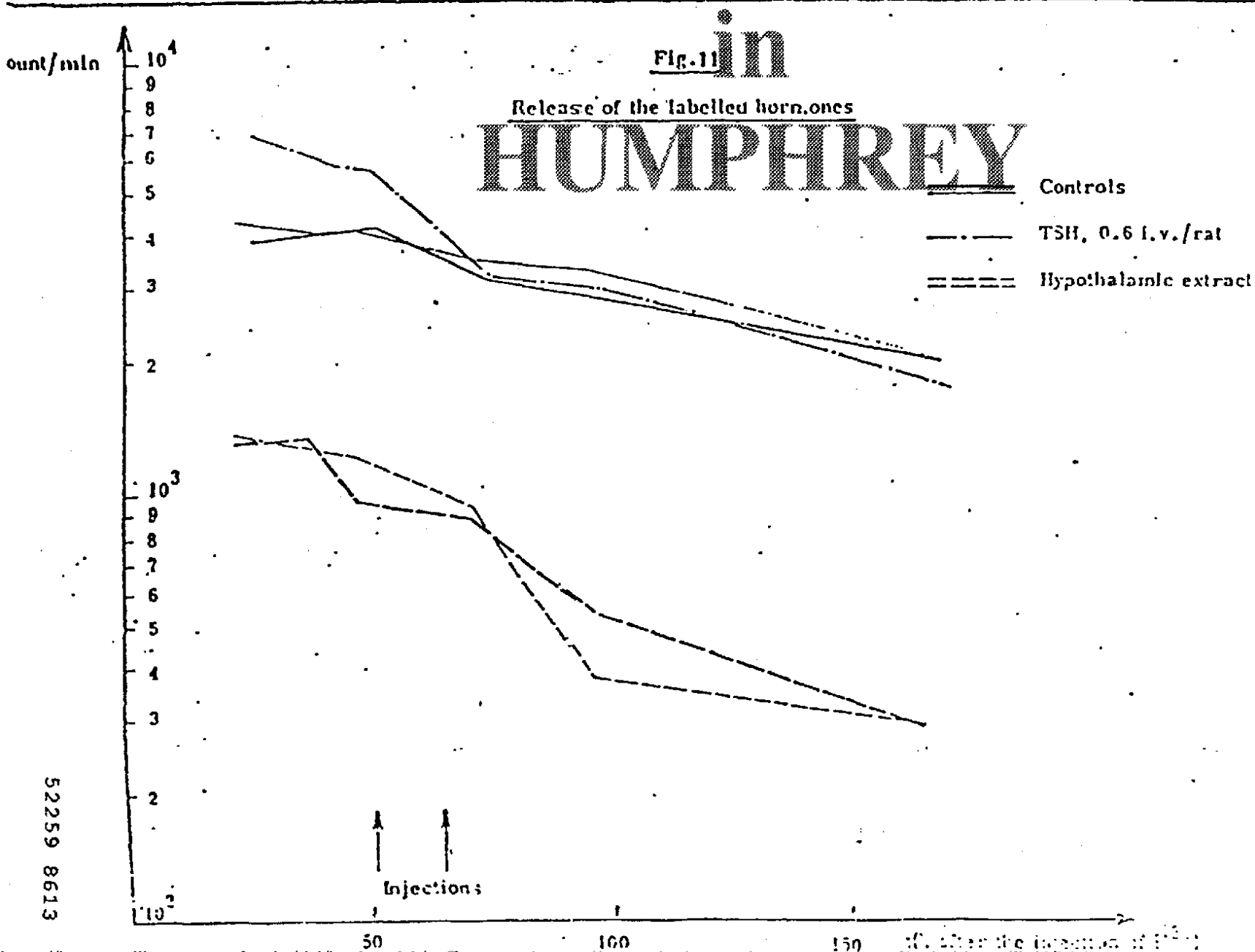
(b) The best quantitative test for the measurement of thyrotropin and thyroid functions is, in our opinion, that which was developed by OVERBEEK et al.<sup>(10)</sup>. This test is based on the fact that the amount of radio-activity measured in the thyroid gland about 24 hours after the administration of  $I^{131}$  to rats reflects the iodine trapping activity of the gland. This activity (i.e. the amount of radio-activity present in the thyroid gland) is much enhanced by the administration of pituitary thyrotropin.

Overbeek et al. have found that the normal thyroid activity can be lowered, and therefore the test made more sensitive, in feeding iodinated casein to the test rats.

We used this test to investigate more thoroughly a possible action of nicotine and of the catecholamines.

(10) OVERBEEK G.A. et al., Acta Endocrinol. 14, 285, 1953.

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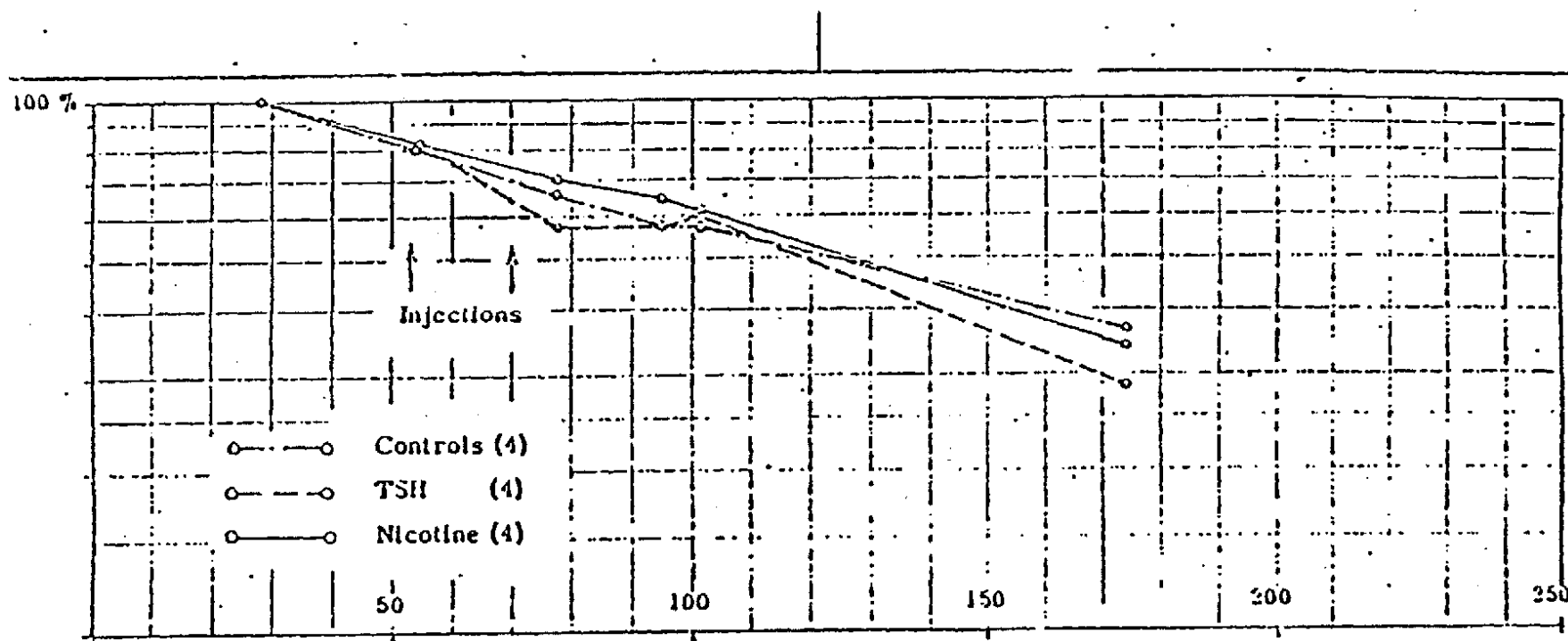
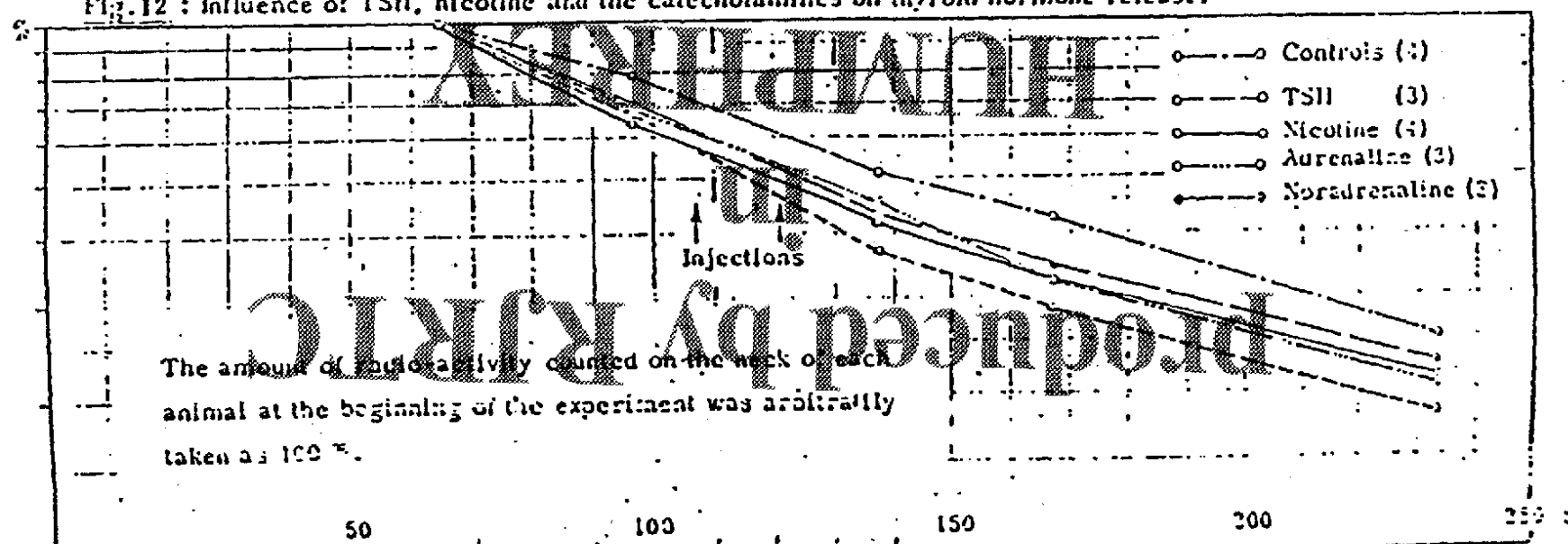


Fig. 12 : Influence of TSH, nicotine and the catecholamines on thyroid hormone release.





Our experimental conditions were as follows:-

Rats of both sexes were fed for 3 days with a diet enriched with iodinated casein. They were given the drugs to be assayed (controls received saline) on day 8 of the special diet. On day 9 the animals received  $0.25 \mu\text{Ci } ^{131}\text{I}$ /rat. On day 10, exactly 24 hours after the administration of  $^{131}\text{I}$ , the rats were killed by ether, and the thyroids were taken out.

The radio-activity was counted direct on the thyroids immediately upon removal of the gland from the body (Well-type scintillation counter).

We first checked the response of our rats to thyrotropin (TSH) and to a hypothalamic extract. A typical response is given in Fig. 13. The actions of 0.15 and 0.60 Int. Units of TSH and of 1  $\gamma$  hypothalamic extract were statistically significant.

We then assayed the action of nicotine and of the catecholamines in this test.

The results of two experiments are gathered in Fig. 14. There is no action of any of the 3 drugs on iodine trapping.

## 2. STATISTICAL RESULTS

### Exp. 1

TSH > controls ( $\alpha_{2-t} \approx 10^{-5}$ )

Nicotine : little or no difference ( $\alpha_{2-t} \gg 10\%$ ).

### Exp. 2

TSH > controls ( $\alpha_{2-t} \approx 10^{-5}$ )

Nicotine : little or no difference ( $\alpha_{2-t} \gg 10\%$ ).

Adrenaline : little or no difference ( $\alpha_{2-t}$  not far from 100 %).

Nor-adrenaline : Tendency towards treated > controls ( $\alpha_{2-t} \approx 14\%$ ).

Fig. 13

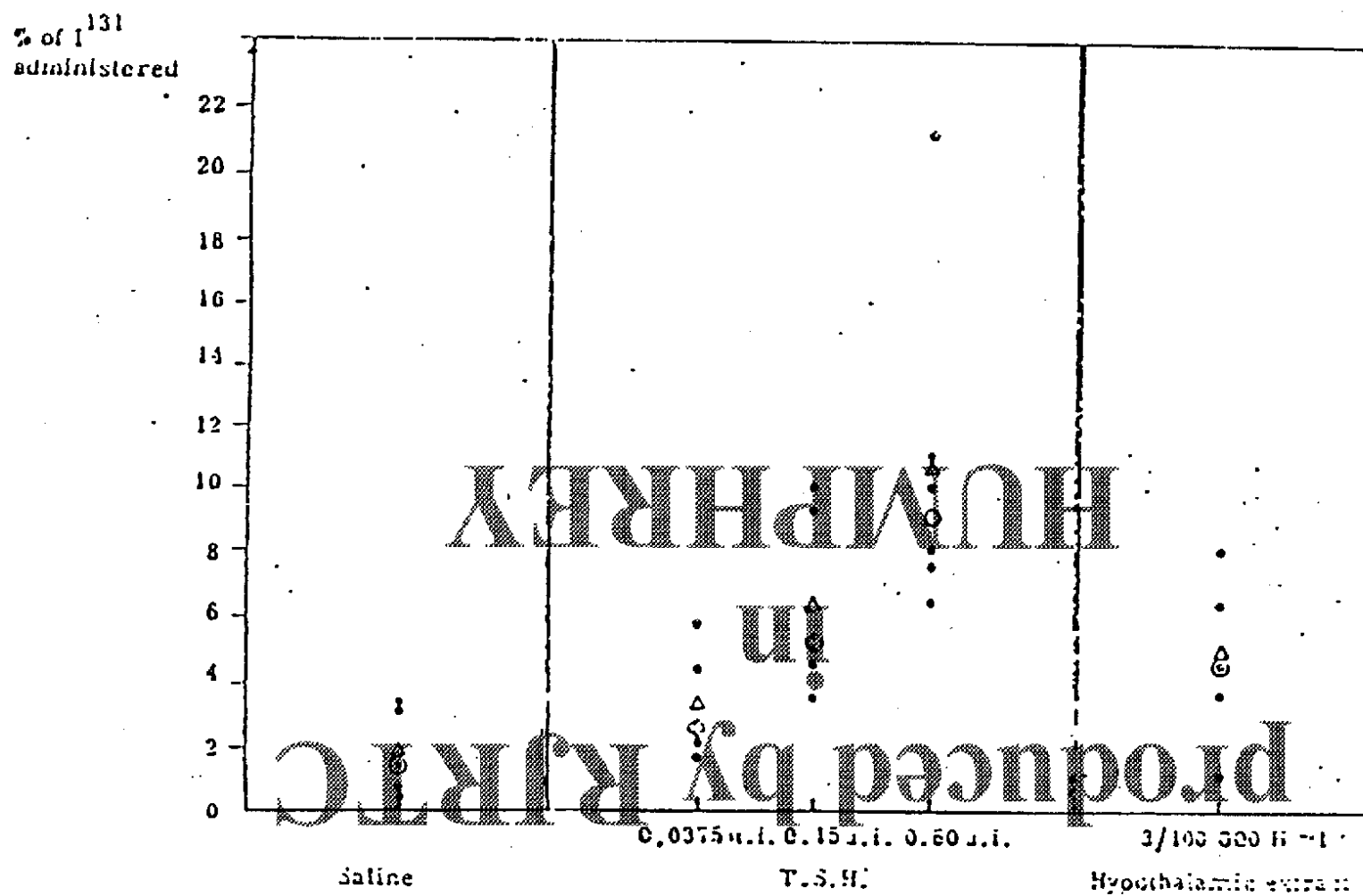
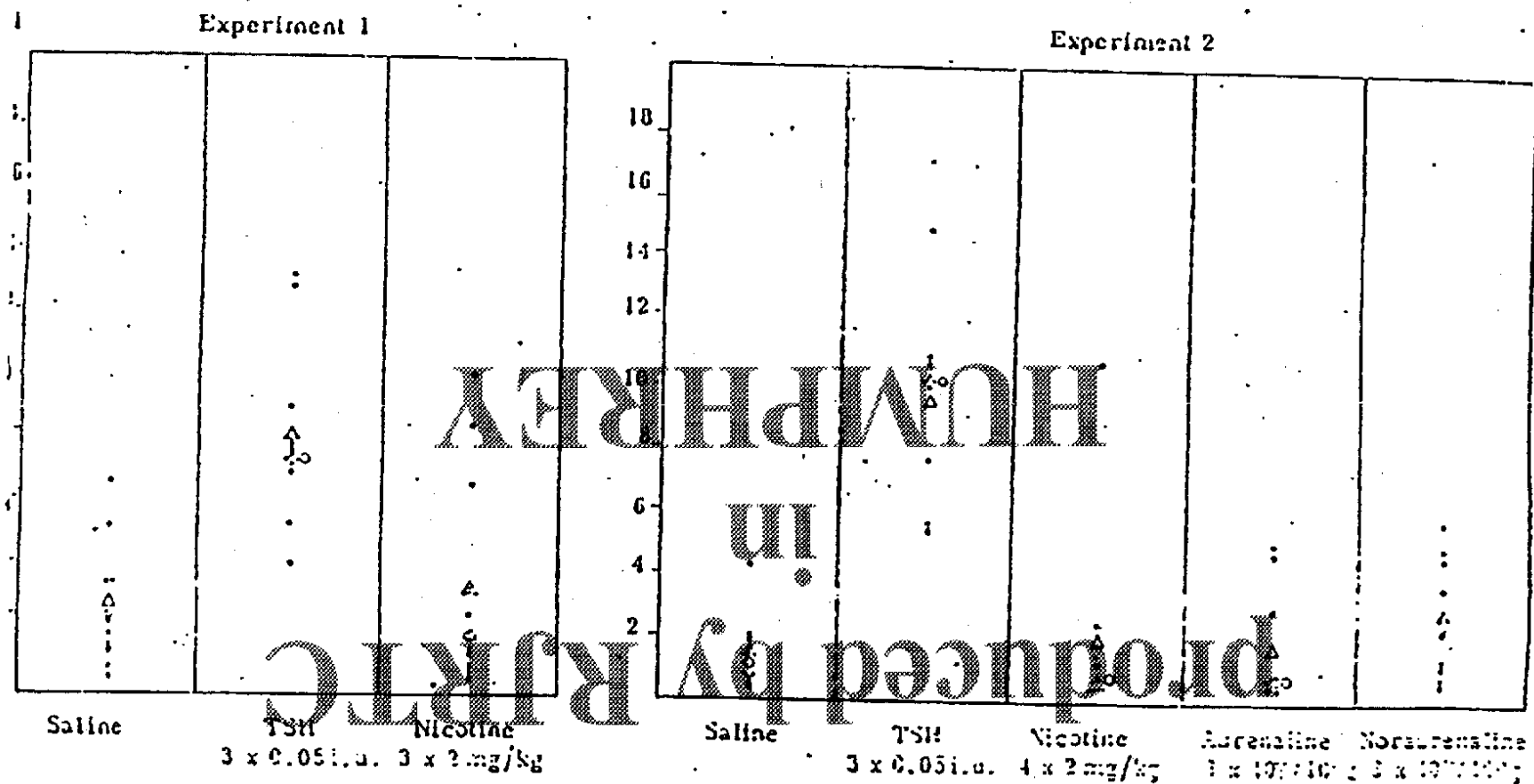
 $I^{131}$  uptake by the thyroid gland

Fig. 14

Iodine uptake by the thyroid of rats in the test of OVERBEEK



We may therefore assert that neither nicotine nor-adrenaline and nor-adrenaline stimulates the release of TRF by the hypothalamus.

### B. Investigation of a Possible Effect of Nicotine on Gonadotropic Control

A possible influence of nicotine on the pituitary gonadotropic activity was investigated.

It is well known that hypothalamus controls this pituitary activity, most probably by way of the discharge of a special factor, which is not already purified.

From another point of view, some authors<sup>(19)</sup> have shown that, in chronic administration, nicotine could lead to a diminished gonadotropic activity.

Our investigations were performed on male and female rats chronically treated.

#### 1. GONADOTROPIC ACTIVITY IN FEMALE RATS

Fifteen female rats received 2 x 2 mg/kg of nicotine per day (5 days a week), the treatment beginning while the body weight averaged 140 g (about two months of age).

They were assayed for gonadotropic activity from the 10th to the 14th week of treatment. The test consisted in determining the cornified cells visible in vaginal smears; the presence of such cells is the signal of a normal oestrus, which lasts about one day and reappears every 4 - 5 days in the rat.

(19) NAKASAWA R., Der Einfluss der chronischen Nicotinvergiftung auf die Funktion der Geschlechtsorgane der weiblichen Ratten. Jap. J. Med. Sc. (Pharmacol.) 5, 109, 1931.

The index "cornified cells per rat per day" was calculated every week during the test period.

As the Japanese authors had shown an inhibition of ovulation by nicotine upon the administered dose being augmented every week, we tried to reproduce this experiment.

Table 9 groups our results.

Table 9

Action of Nicotine on the Female Cycles

	Nicotine mg/kg/day	Oestrus index (cornified cells/ rat/day)	Observations
Controls	0	0.250	normal cycle
10th week of treatment	4	0.376	prolonged oestrus (one gonadotropic hormone blocked)
11th "	8-12	0.320	
12th "	12-16	0.184	
13th-14th week	16	0.230	oestrus rarified (both gonadotropic hormones blocked)
			return to normal cycles

This table allows the following observations to be made:-

- (a) After a long-lasting administration of large doses of nicotine (4 mg/kg/day), the female cycle was disturbed in the sense of a prolonged oestrus. This fact means that there was no ovulation, probably owing to a blockade in the release of the gonadotropic hormone called "luteinising hormone".

- (b) When the dose of nicotine was continually augmented every week until the enormous dose of 16 mg/kg/day was administered, both gonadotropic hormones were blocked, and some of the rats showed no more oestrus and no cycle at all.
- (c) However, this enormous dose - which, for a woman weighing 60 kg, would correspond to about 450 cigarettes a day - was not sufficient, if not augmented once more, to maintain the disturbances in the female cycle.

## 2. GNADOTROPIC ACTIVITY IN MALE RATS

The weights of testes and seminal vesicles of 105 chronically treated and control male rats were measured.

Table 10 groups our results; it shows that there is no action on the weights of testes (measurement of spermatogenic activity), and that there is a tendency towards a diminution of the weights of seminal vesicles (endocrine activity), which diminution is significant only after seven months of treatment.

Why doesn't the 70:1 rule (rats:men) operate here?

I wondered that a dose level of 16 mg/kg for a rat would correspond pharmacologically to a dose of 16 mg for a man.  
But 16 mg/kg/day for a rat would not be the same as the amount of nicotine from 450 cigs per day.

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Table 10

Chronic Action of Nicotine on Gonadotrophic Function in Male Rats

Duration of treatment Organs	2 months		4 months		5 months		6 months		7 months	
	Controls	Nicotine treated	Controls	Nicotine treated	Controls	Nicotine treated	Controls	Nicotine treated	Controls	Nicotine treated
Testes (mean weight in mg)	2957	2686	3601	3501	2395	3096	3067	3620	3312	3103
$\alpha$ e)		> 10 %		> 10 %		> 10 %		no diff.		no diff.
Seminal vesicles (mean weight in mg)	707	563	1161	1100	1247	1102	1391	1116	1209	1023
$\alpha$ e)		> 10 %		> 10 %		> 10 %		> 10 %		5 %

e)  $\alpha$  = probability of error for a difference between treated and control animals

## VI. DISCUSSION AND GENERAL CONCLUSIONS

One of the reasons for smoking could be represented by the great help that nicotine brings to the mastering of stressful situations. Of course, there are other possible reasons, and the main other one is perhaps that which has been studied by Prof. J.H. Burn<sup>(20)</sup>: "a stimulation when we wish to concentrate attention on a problem". Prof. Burn has shown that this greater capacity to concentrate attention is most probably due - as well as the vaso-constrictive activity of nicotine - to the liberation of nor-adrenaline from hypothalamus that is brought about by nicotine administration.

Our investigation of various hypothalamic functions in which nicotine seems to interfere has shown a discrepancy between the responses to nicotine of these various functions, as concerns a possible effect of nor-adrenaline release, although these responses are very similar in their threshold values.

1. The antidiuretic effect of nicotine as well as its antiappetite (or anorexic) effect are not influenced by a pretreatment of the test animals with reserpine, after the administration of which there remains no more available nor-adrenaline in the tissues. It seems, therefore, that nicotine does not act, in both tests, by way of nor-adrenaline release from hypothalamus or other tissues.
2. The stimulation by nicotine of the corticotropic function seems to be partly inhibited after the release of nor-adrenaline owing to reserpine.

(20) BURN, J.H. : The action of nicotine and the pleasure of smoking. The Advancem. of Sci., 17 (70), 494 (1961).



Nicotine still acts in this test after complete nor-adrenaline release, but its effect, compared to that of nicotine alone, is markedly less pronounced.

The antidiuretic, the reaction to stress, the regulation of appetite, the mobilization and degradation of lipids are all effects which are mediated by the hypothalamus itself.

We therefore wanted to ascertain:-

- (a) Whether or not the other main hypothalamic activities (thyrotropic regulation and gonadotropic regulation) are affected by nicotine administration. A thorough investigation of both activities has shown that nicotine does not act at all upon thyrotropic regulation, and that it acts very slightly, and only so if enormous doses are employed, upon gonadotropic regulation.
- (b) Whether or not nicotine still interferes in the hypothalamic activities when the corresponding centres are destroyed. The hypothalamus is formed of groups of cells called "nuclei"; some of them have been related to one or the other of the numerous hypothalamus activities, and are called the "centres" of these activities. There are generally two centres governing one effect: a stimulating centre and an inhibiting centre; however, in most cases only the position of the stimulating centre is well known. If nicotine acts on this stimulating centre, it must act no more when the centre is destroyed.

This experiment was made in three cases:-

- (i) The centre that synthesizes the antidiuretic hormone was destroyed by electro-coagulation; nicotine still acted after this destruction.

- (ii) The centre that stimulates satiety was destroyed in the same way; nicotine still acted after this destruction.
- (iii) Morphine is said to inhibit the centre that stimulates the corticotropic function; nicotine acted less markedly with morphine administration than without it.

It seems, therefore, that nicotine does not stimulate the hypothalamic centres related to its effect. It is possible that nicotine, being an artificial drug, bears inhibiting activities - and not stimulating ones - on the antagonistic centres. Unfortunately it is almost impossible to study this hypothesis for the moment, as the exact locations of the inhibiting centres are not clearly known.

From all our data it seems that the effect of nicotine in the "stress reaction" is a very prominent and a very complicated one.

The understanding and thorough investigation of this effect seems of the greatest importance : it is by this very effect that nicotine acts as a "tranquillizer".

Geneva, 2.2.1962

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REPORT No 1  
regarding  
PROJECT HIPPO II

by

O. Libert

INTRODUCTION

To investigate whether or not nicotine acts on brain functions in a way similar to that of the tranquilizing drugs, it was necessary to make a brief survey of the literature as regards the mode of action of these drugs.

In this first Report we endeavour to summarize the numerous investigations that have been made on this subject, and the hypotheses to which they have led.

As nicotine seemed to interfere in the hypothalamic reaction to stress, we thought it necessary to investigate also in the literature whether or not this hypothalamic function is dependent upon other parts of the brain. The summary of data found in the literature about such a regulation is given in the second part of this Report.

A final discussion introduces our research programme as established in our Proposal.

## I. MODE OF ACTION OF TRANQUILIZING DRUGS

### A. Definitions and Pharmacological Activities

In 1958 Himwich<sup>(1)</sup> defined this new group of drugs in the following terms:

"The last few years have seen the increasing use of a new group of drugs effective for overactive psychotic patients and presenting interesting and important differences from procedures and drugs previously employed in the management of individuals with such behavioral disorders. In fact, in order to give an appropriate name to these drugs, it was necessary to invent a nomenclature. Many terms, all emphasizing one or another aspect of the actions of these drugs have been devised. The one most frequently used is tranquilizer, indicating a sedative or calming effect without enforcement of sleep. Others are ataraxic, denoting peace of mind, and neuroleptic and neuroplegic, indicating diminutions in the intensity of nerve function." ...

"The chief usefulness of the new drugs, which at present lies in the field of psychiatry, is being extended into the many medical and surgical specialties. It is true that a drug not only changes favorably the malfunction of the organ for which it is intended but also acts, perhaps to a lesser extent, on the entire body. Either indirectly, by their influence on the brain, or by their influence on the peripheral nerves, the new drugs exert potent effects on the viscera of the body, including the gastrointestinal tract and the cardiorespiratory system and the endocrine glands."

(1) H. E. Himwich, Science, 127, 59, (1958).

Tranquilizers are mainly applied in psychiatry to schizophrenic, hyperactive or psychoneurotic patients. However, "these drugs also exhibit decided usefulness for the members of our so-called normal population who are subjected to intolerable stress. A businessman with a demanding and unreasonable supervisor or a woman with insufficient funds to run her home according to her ideal standard can gradually build up an emotional impasse so that perspective is lost, as the darker side of the situation is increasingly magnified, until a state of panic may develop. Restoration to a more objective evaluation of the situation may be secured by psychotherapeutic discussions with a physician, and an important action of tranquilizing drugs is to render the patient more receptive to other kinds of therapy. In fact, at times a psychopharmacologic drug may be essential for a successful psychiatric interview. Ephemeral disturbances - for example, the anxiety and tension aroused by an impending surgical operation - can be pleasantly dissipated by a small dose of a tranquilizing agent." (1)

The most generally used neuroleptic drugs are reserpine (alkaloid obtained from Rauwolfia) and chlorpromazine (synthetic chemical belonging to the phenothiazine group, also called Largactil).

#### B. Mode of Action

The mode of action of tranquilizers in man is entirely unknown. However, animal experimentation has shown that their tranquilizing activity seems to be accompanied by some measurable effects:-

1. Effects on brain electrical activity.
2. Effects on autonomic functions within the central nervous system and on the stores of neuro-humoral agents in the brain.
3. Interference with the hypothalamic reaction to stressful stimuli.

These data have led to hypotheses that still remain to be proved.

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### 1) Effects on brain electrical activity

The tranquilizers affect the electrical activity of the subcortical structures regarded as parts of the anatomical substrate of emotion (Fig. 1) :-

- The midbrain reticular formation,
- The hypothalamus,
- The components of the rhinencephalon.

These three brain regions are connected with each other, and all with the cortex. They are phylogenetically very ancient parts of the central nervous system and may be called the "primitive brain".

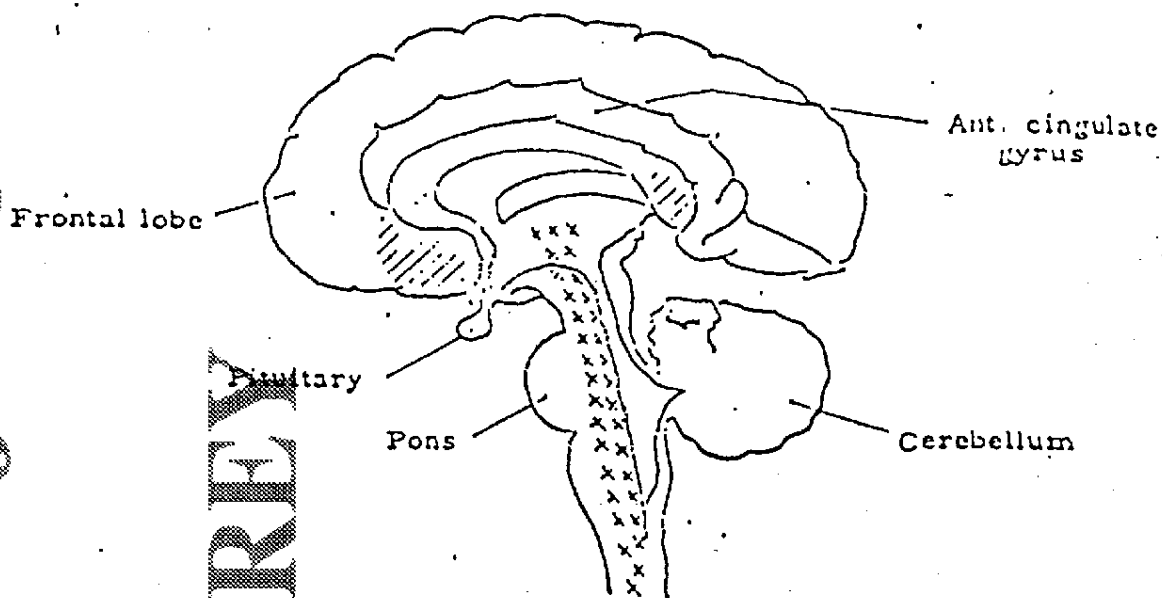
- (a) The midbrain reticular formation was studied by Magoun and his co-workers<sup>(2)</sup>, who showed that this system, together with the diffuse thalamic projections, "affords the emotional cloak which accompanies some kinds of stimulation of the body"<sup>(1)</sup>.

Particular stimuli are related to changes in the electro-encephalogram of the reticular formation, forming the "alert pattern", very different from the high-amplitude waves characteristic for the "rest-pattern". Chlorpromazine blocks the reticular formation, which no longer shows the alert-pattern. Reserpine, on the contrary, stimulates the midbrain reticular formation activity, and produces the alert-pattern. It must be noticed that chlorpromazine exerts some anaesthetic properties, while reserpine does not. The tranquilizing effect of this latter product must act on other parts of the brain.

(2) H.W. Magoun : The Waking Brain, C.C. Thomas, 1958.

Fig. 1

a) Medial sagittal section of human brain

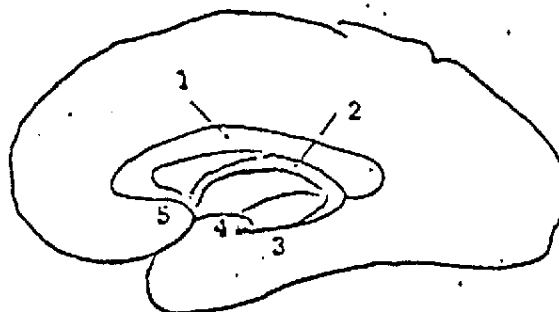


Midbrain Reticular Formation

Hypothalamus

Rhinencephalon (limbic system)

b) Medial surface of the cerebral hemisphere, showing the different parts of the rhinencephalon



1 Corpus callosum

2 Fornix

3 Hippocampal region

4 Uncus

5 Amygdala

- (b) The hypothalamus, out of its "neuro-endocrine" functions, "organizes the visceral manifestations which are overactive in disturbed patients" (1).

Reserpine and chlorpromazine both inhibit electrical activity of hypothalamic structures, particularly those of the posterior nuclei which regulate the sympathetic nerves of the viscera and blood vessels.

- (c) "The part of the brain concerned especially with emotional experience and the one least understood is the rhinencephalon, which is also called the limbic system or the visceral brain" (1).

One part of the rhinencephalon, called the amygdala, is that part of the brain which is most susceptible to reserpine and chlorpromazine. Tranquilization is associated with amygdaloid hyperactivity, which can be recorded on electro-encephalograms. After a therapeutic dose of chlorpromazine, a cat remains indifferent in front of a mouse placed in its cage, while convulsive waves are disclosed in the amygdala.

#### 2) Effects on neuro-humoral agents

Several groups of workers have shown that the brain stores in catecholamines (adrenaline and noradrenaline) as well as in serotonin (5-hydroxytryptamine) are depressed soon after the administration of reserpine to a rabbit (3)(4)(5)(6).

- (3) Å. Bertler, Acta Physiol. Scand., 51, 75 (1961).  
(4) P.A. Shore et al., N. Y. Acad. Sci., 66, 417 (1957).  
(5) B.B. Brodie et al., Science, 125 (3261), 1293 (1957).  
(6) A. Pletsher; P.A. Shore and B.B. Brodie, J. Pharmacol. exp. Therap., 115, 84 (1956).

In the rabbit, the time-curves are very similar for both groups of the brain amines (Fig. 2).

In the rat, they are slightly different: the noradrenaline concentration was found to be depressed by reserpine more rapidly than the serotonin concentration <sup>(7)</sup>.

The depressing action of reserpine on the brain amine stores is very long-lasting, and the return to normal concentrations takes about one week.

This similarity in the depletion of serotonin and adrenaline can be compared with the similarity of the areas of distribution of these two neuro-humoral agents in the various parts of the brain <sup>(1)</sup>. They are both at their highest concentration in the hypothalamus.

These two facts very probably are related to similar metabolic pathways: serotonin and the catecholamines as well are metabolized by the same enzyme, monoamine oxidase; the areas of distribution of this enzyme in the brain are also superimposed to those of the catecholamines and of serotonin.

Reserpine seems to interfere with the organic binding of the amines into the brain cells. The latest and more probable hypothesis is that of Brodie and his co-workers <sup>(8)</sup>; normally serotonin would be bound with some constituents of the brain cells in two forms: one labile form, and the other one more stable (cytoplasmic granules); the labile bond would be broken by reserpine, and the free serotonin would be immediately metabolized by the enzyme monoamine oxidase, always present

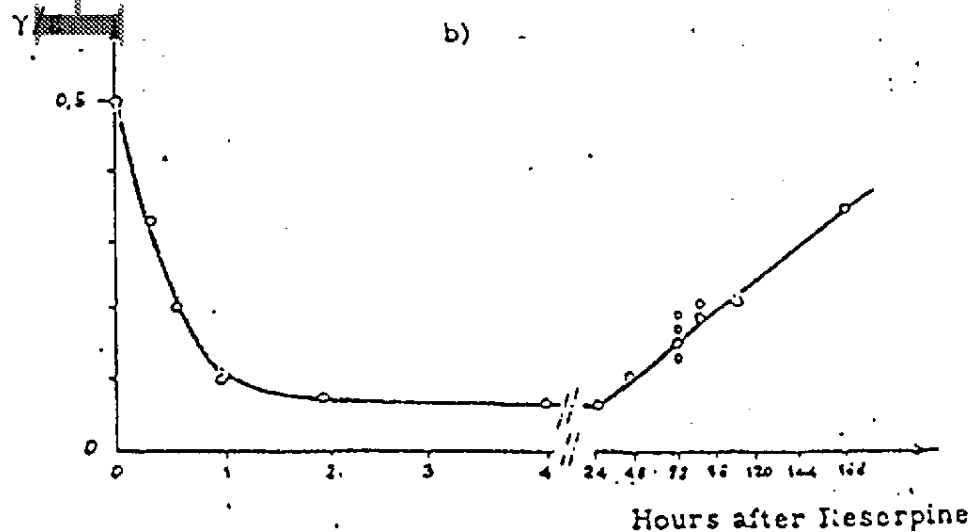
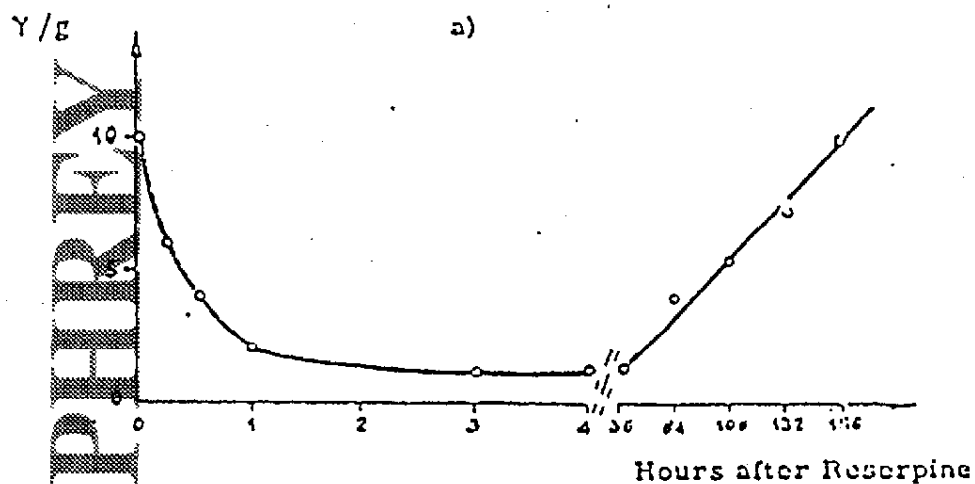
(7) N. T. Kärki and M. K. Paasonen, *J. Neurochem.*, 3, 352 (1959).

(8) E. Costa; G. L. Gessa; C. Hirsch; R. Kuntzman and B. B. Brodie, *Ann. N.Y. Acad. Sci.*, 96, 118 (1962).

in

Fig. 2

Concentration of Serotonin a) and of Noradrenalin b) in the brain of a rabbit at various times after intravenous administration of Reserpine.



in the same cells as serotonin itself. The metabolite, 5-hydroxyindole acetic acid (5-HIAA), is actually increased in urine after reserpine administration <sup>(9)</sup>.

With this hypothesis in mind, the effects of the drug reserpine would seem to be due in part to the depression in the stores of the brain amines.

As regards chlorpromazine, its mode of action seems different, although it was shown that chlorpromazine is able to depress the body stores of serotonin (mainly in the blood platelets), even more rapidly than does reserpine <sup>(10)</sup>.

What would be the physiological meanings of the depletion of the brain amines following the administration of reserpine? We can only answer for the moment by hypotheses.

#### (a) Physiological activities of the brain amines

Brodie and his co-workers <sup>(8)</sup>, after Gaddum et al. <sup>(11)</sup>, think that serotonin is actually a "central neuro-hormone"; they give some evidence of this hypothesis, although no rigorous proof has been advanced so far.

Marazzi and Hart <sup>(12)</sup> "propose a theory depending upon an equilibrium between various hormones...." They contrast the stimulating effects of acetyl-choline, a chemical mediator of the nerve impulse across the synapse, the junction between two ner-

(9) F.M. Berger, Ann. N.Y. Acad. Sci., 66, 686 (1957).

(10) G. Bartholini et al., *Experientia* 17, 541 (1961).

(11) A.J. Amin; T.B.B. Crawford and J.H. Gaddum, *J. Physiol.* 126, 596 (1954).

(12) A.S. Marazzi and E.R. Hart, in Himwich: *Tranquillizing drugs* (1957).

ves, with the inhibiting action of adrenaline"... "Their experimental work also discloses that the depressant influence of serotonin is more potent than that of adrenaline". (1)

In other words, the normal behaviour would depend upon an equilibrium between two groups of "central neuro-hormones":-

1 - acetyl-choline, the chemical transmitter of the nervous impulse across the synapse, not only in the parasympathetic system, but also within the central nervous system.

2 - Serotonin and the catecholamines, inhibitors of this same impulse. In the original theory of Brodie, the accent is made upon serotonin only; however, adrenaline and noradrenaline may be as important as serotonin in this phenomenon.

As an extension of the theory, mental derangement would be caused by an imbalance between these various hormones, imbalance which would be improved by the effects of the tranquilizing drugs.

For Woolley and his co-workers<sup>(13)(14)</sup>, this imbalance could be due to an error of serotonin metabolism in the brain. This working hypothesis has not received sufficient proof until now but seems very promising.

(b) Theory regarding the therapeutic effects of tranquilizing drugs

As an attempt to summarize data concerning the biochemical effects of tranquilizing drugs on the brain stores of the neurohormones, we can make the following observations :-

(13) D. W. Woolley and E. Shaw, Brit. Med. J. ii, 122 (1954).

(14) D. W. Woolley and N. K. Campbell, Ann. N.Y. Acad. Sci. 96, 108 (1962).

i - Serotonine as well as the catecholamines are depressed in the body after the administration of reserpine; the time-curves of their disparition from the brain are quite similar for both groups of neurohormones.

ii - Serotonine and the catecholamines seem to inhibit the transmission of the nerve impulse in the brain synapse, acetylcholine being on the contrary the mediator of this transmission.

iii - The tranquilizing effects of reserpine could be partly due to the observed depletion in the inhibiting amines, thus leaving the acetylcholine activity unopposed. Some of the pharmacological effects observed after the administration of reserpine are actually signs of acetylcholine activity.

iv - From another point of view, the depletion of the amines from the body stores deprives the organism of part of the mechanism of reactivity, thus decreasing the manifestations of anxiety and tension.

### 3) Interference with the hypothalamo-pituitary-adrenal axis

Reserpine and chlorpromazine, like other drugs acting as depressants of the nervous system (morphine, barbiturates), tend to prevent the extra-release of ACTH following stressful stimuli <sup>(15)</sup>. Sayers et al. (cited in 15) think that several of these drugs depress the reticular activating system and thus prevent stimuli that release the CRF from reaching the hypothalamus (see part II).

(15) R. Gaunt et al., Science 133, 613 (1951).

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However, Brodie (cited in 15) found that only long-continued reserpine treatment leads to greatly reduced levels of pituitary ACTH, and considers any apparent inhibition of secretion to be rather a manifestation of exhaustion.

Some other data seem to agree with this view: Khazan et al. (16) showed that an acute treatment by reserpine produced an activation of the pituitary-adrenal system (adrenal hypertrophy, ascorbic acid depletion and increase in adrenal corticosteroids). These modifications were no longer observed after a prolonged treatment.

Stockham et al. (17) have shown that corticosterone concentration in plasma is greatly increased two hours after one injection of reserpine. This increase lasts some 14 hours, and the normal level is not yet obtained after 24 hours. Reserpine acts no more after hypophysectomy. A prolonged treatment (10 days) increases the corticosterone level in blood only with very high doses. Guillemin (18) found no activity at all for treatments lasting four days or more. Serotonine itself, which is released from its stores by reserpine, acts as a "corticotropin-releaser", and this effect is mediated via the hypothalamus, for it can no more be observed after lesioning of the "corticotropin-centres" (19).

Thus it seems that tranquilization may be due in part to an interference with the hypothalamo-pituitary-adrenal axis. For a unique dose the drug may enhance the CRF\* release by the hypothalamus. If the

(16) N. Khazan et al., Proc. Soc. exp. Biol. Med. 106, 570 (1961).

(17) R. Montanari and M. A. Stockham, J. Endocr. 24, IX (1962).

(18) R. Guillemin, in "Brain mechanisms and drug action", p. 99, (1957).

(19) P. G. Smelik and P. de Wied, Experientia 14, 17 (1958).

\* CRF = corticotropin-releasing-factor, secreted by the hypothalamus.

treatment is very long-lasting, it may produce an exhaustion of the production of the pituitary corticotropic hormone, which exhaustion is so pronounced as to prevent the extra-release following a stressful stimulus.

It is possible that this manifestation of reserpine activity may be mediated through depletion of serotonin stores in the cells.

## II. INFLUENCES OF OTHER PARTS OF THE BRAIN UPON HYPOTHALAMUS IN THE CORTICOTROPIN- REGULATION MECHANISM

The "primitive brain" that is specially affected by the tranquilizing drugs seems to be also related to the corticotropin-regulation mechanism.

### A. Midbrain

Anderson<sup>(20)</sup> has shown that transection of the brain stem at the midbrain level induced in dogs a nearly total suppression of the corticotropin effect normally following stressful stimuli, as measured by the excretion of urinary steroids. The "midbrain dogs" showed no increase in the urinary steroids after a series of severe stresses (mainly painful ones), contrary to the case of normal dogs, although neither the pituitaries nor the hypothalami of the operated dogs showed any lesions. This observation indicates that the midbrain plays a vital rôle in mediating corticotropin release.

(20) E. Anderson et al., Rec. Progr. in Hormone Res. 13, 21-59 (1957).

It seems that the disturbances in the endocrine reaction to stress are due to interruption of those ascending fibres terminating in the mid-brain reticular formation which are known to conduct pain impulses to subthalamie and thalamic nuclei. It is possible that impulses propagated by them may reach the hypothalamus through multi-synaptic pathways.

It is known that midbrain nuclei innervate the hypothalamus. These nuclei are always severed while transecting the midbrain.

The observation to be made after Anderson's experiments is that the lesion performed in the midbrain destroyed a neural mechanism that has the capacity of activating the hypothalamus-pituitary system

Redgate (21) has similarly shown a decrease in the corticotropin release after a stress, when spinal cord was sectioned. His experiments clearly show the localization of an ascending spinal pathway mediating the endocrine reaction to stress, situated in a region rostral to the pons (see Fig. 1).

#### B. Higher brain level

Porter (22) was the first to show a functional relationship between the hypothalamus and higher brain levels. He thinks that hypothalamus is activated by a direct nervous way.

His experiments (administration of adrenaline, which produces a "stress-reaction", electrical stimulation or lesions) have shown various such relationships (Fig. 3).

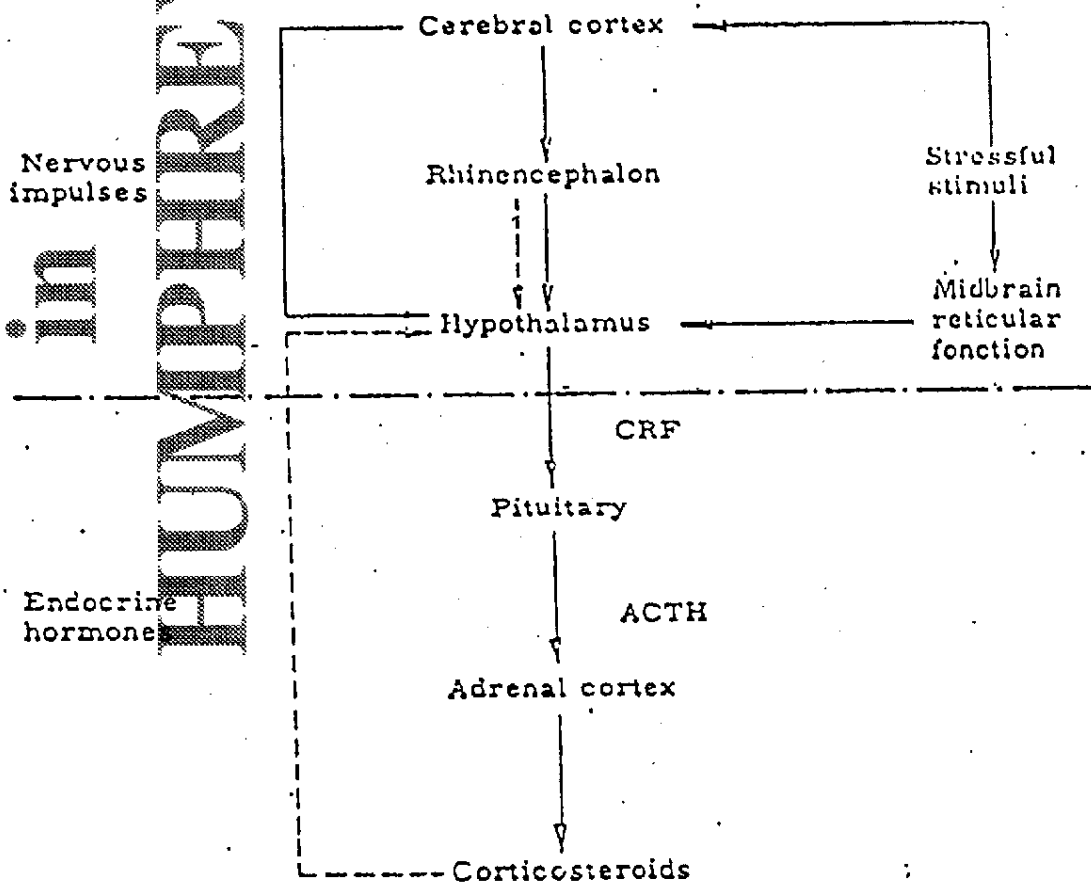
(21) E.S. Redgate, Proc. Soc. exp. Biol. Med. 105, 528 (1960).

id. , Endocrinol. 70, 263 (1962).

(22) R.W. Porter, Rec. Progr. in Hormone Res. 10, 1-27 (1954).

Fig. 3

Schematic representation of nervous and endocrine control of reaction to stressful stimuli



—●— Stimulation  
 - - - - - Inhibition

- (a) Some parts of the cerebral cortex (mainly the anterior cingulate gyrus and the orbital surface of the frontal lobe) exert excitatory influences on the hypothalamo-corticotropin stimulating function.
- (b) Inhibitory influences were exerted by stimulation of the hippocampal region mainly by the uncus.

These primary results were extended by other authors : Mason<sup>(23)</sup> found a marked facilitating of corticotropin release following amygdaloid stimulation in monkeys.

Slusher and Hyde<sup>(24)(25)(26)</sup> have demonstrated in cats the existence of inhibitory influences on adrenal corticosteroids excretion, by stimulation of various structures of the limbic system (rhinencephalon).

To summarize these very important investigations, we can make the following observations on the influences of other parts of the brain upon the hypothalamic function in the response to stressful stimuli :-

i - Nervous influences are of fundamental importance. They seem to precede and to condition the neuro-humoral release of CRF by the hypothalamus.

ii - The existence of an activating neural mechanism located in the midbrain has been clearly demonstrated; another one was found in some cortical areas.

(23) J. W. Mason, Am. J. Physiol. 196, 44 (1959).

(24) M. A. Slusher and J. E. Hyde, Federation Proc. 19, 292 (1960).

(25) id. id. , Endocrin. 68, 773 (1961).

(26) id. id. id. 69, 1080 (1961).

iii - Inhibitory and facilitatory influences as well can be demonstrated in the limbic system (also called rhinencephalon) which is related to emotional behaviour.

iv - The stimulating structures must be functionally intact in order to allow a normal endocrine reaction to stress.

### DISCUSSION

Faced with a disturbing situation - whatever its cause may be - our organism reacts in a very primitive way, entirely independent of our will. This reaction is produced by two kinds of phenomena: first, the mobilization of the neuro-vegetative system; second, the stimulation of the hypothalamo-pituitary-adrenal endocrine system.

This "alarm-reaction", which can be called "non-specific" for it is independent of the nature of the stressful stimulus to which it responds, is the preparation of our organism to escape the disturbing cause. Such a reaction bears many unpleasant manifestations: acceleration of the pulse, shivering, etc., which are signs of the neuro-vegetative hyper-activity.

The endocrine part of the reaction supplies to the cells the compounds necessitated in greater amounts by their enhanced activity. This is not entirely beneficial however; if repeated very frequently and for a long period, the development of pathological manifestations like atherosclerosis, or even some kinds of cancer, could be enhanced.

What are the effects of pharmacological tranquilization? The new drugs mainly inhibit the neuro-vegetative part of the alarm-reaction, thus alienating its unpleasant manifestations.

They seem to facilitate at least for a unique administration on the contrary the endocrine part of the alarm reaction, i.e. the stimulation of the hypothalamo-pituitary-adrenal axis. However, a prolonged treatment shows no more facilitating effect, and even exhaustion can appear.

Is it possible to perceive any similarities between these effects of the new drugs called "tranquillizers" and the feeling of comfort experienced by a smoker ?

Some of these similarities appear at first sight and are certainly worth while studying :-

1. Nicotine, like reserpine itself, releases noradrenaline from its stores; it seems also to release serotonin stores from the brain <sup>(27)</sup>.
2. The administration of one dose of nicotine enhances the hyperproduction of corticosteroids in the adrenal consecutive to a stress, as does reserpine itself.

It is certainly of great importance to study correlatively and as a function of time the effects of both nicotine and reserpine on the concentration in brain and in blood of the three groups of substances :-

- (a) adrenaline and noradrenaline (catecholamines),
- (b) serotonin,
- (c) corticosteroids or corticotropin.

(27) H. G. Boit and B. Mehlis, Die Natur Wissensch. 48, 602 (1961).

RESEARCH PROGRAMME

The research programme established in our Proposal will be followed.

Investigations of the new determinations to be performed (serotonine, catecholamines) are in progress.

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*March*  
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FINAL REPORT  
on  
PROJECT HIPPO II

by

C.H. Haselbach and O. Libert

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SUMMARY AND DISCUSSION

The aim of the whole research "HIPPO" was to understand some of the activities of nicotine - those activities that could explain why cigarette smokers are so fond of their habit. It was also our purpose to compare these effects with those of the new drugs called "tranquillizers", which might supersede tobacco habits in the near future. We studied mainly the drug called reserpine.

Why does one smoke? It is certainly not because of nicotine's cardio-vascular activities, which are so well-known to pharmacologists. The reasons for the "pleasure of smoking" must be found partly in the relief of anxiety that cigarette smoking brings so constantly, and in such a very short time.

This sedative - or soothing - effect of cigarette smoking and of nicotine is however very different from the "tranquillizing" effect as it was defined by pharmacologists after the discovery of the

Rauwolfia alkaloids. Tranquillizers are highly effective in the management of overactive psychotic patients and, as such, are largely used in psychiatry; nicotine is certainly devoid of such effects.

However, as the new drugs are used increasingly by "the members of our so-called normal population who are subjected to intolerable stress" (see our First Report on ILLINO II, p. 3), they might, from this point of view, supersede tobacco habits.

Our investigation definitely shows that both kinds of drugs act quite differently, and that nicotine may be considered (its cardiovascular effects not being contemplated here) as more "beneficial" or less noxious - than the new tranquillizers, from some very important points of view.

The so-called "beneficial" effects of nicotine are of two kinds:

1. Enhancing effect on the pituitary-adrenal response to stress;
2. Regulation of body weight.

These effects do not seem to be shared by reserpine, which on the contrary shows undesirable side-actions that are not given by nicotine, i.e. a nearly complete blockade of gonadic and thyroid activities, reflecting most probably a general blockade of the hypothalamo-pituitary system, which normally controls all the endocrine activities.

#### 1. Enhancing Effect of Nicotine on the Pituitary-Adrenal Response to Stress

The normal reaction of an organism to a stressful situation - either psychological or physiological in nature - is a stimulation of the pituitary-adrenal function (adreno-corticotrophic hormone or ACTH), which in turn is stimulated by the so-called "Corticotrophin releasing factor" (CRF) or the hypothalamus.

We observed, by the use of two main tests, a marked stimulation of this natural response to stress, when rats were submitted to nicotine administration, either in acute or in prolonged treatment. In our opinion this action could be one of the explanations of the "pleasure of smoking".

## 2. Body Weight Regulation

This effect seems to be a very important point for tobacco manufacturers: nicotine is actually one of the most potent drugs against obesity.

Data presented in our Final Report on HIPRO I showed definitely that nicotine bears a three-fold activity against obesity:

- (a) by its anti-appetite effect,
- (b) in mobilizing the lipid depots and releasing free fatty acid in blood,
- (c) after this mobilization, in enhancing the disappearance from blood of these same fatty acids, possibly by stimulating their degradation.

It thus seems that the pleasure of smoking is actually supported by physiological effects of nicotine that can be considered as beneficial for the organism. Most probably, such beneficial effects are both due to stimulation of adreno-corticotrophin production by the pituitary. Actually, this hormone exerts, by itself, the physiological control of fat mobilization and of food intake as well as of the reaction to stress.

The diagrammatic representation on page 5 is an endeavour to summarize our data.

It emphasizes the specificity of the effects of nicotine on hypothalamic functions, facing the absence of specificity of reserpine effect (blockade of all pituitary activities). Nicotine stimulates the release of vasopressine, oxytocine and CRF, without showing any effect on the other hypothalamic factors affecting various ante-pituitary functions.

Vasopressine, oxytocine and CRF seem to be synthesized in very closed regions of the hypothalamus. From another point of view, it is possible that vasopressine and CRF are related in function or in molecular structure. It would appear that nicotine acts specifically on this function of the hypothalamus.

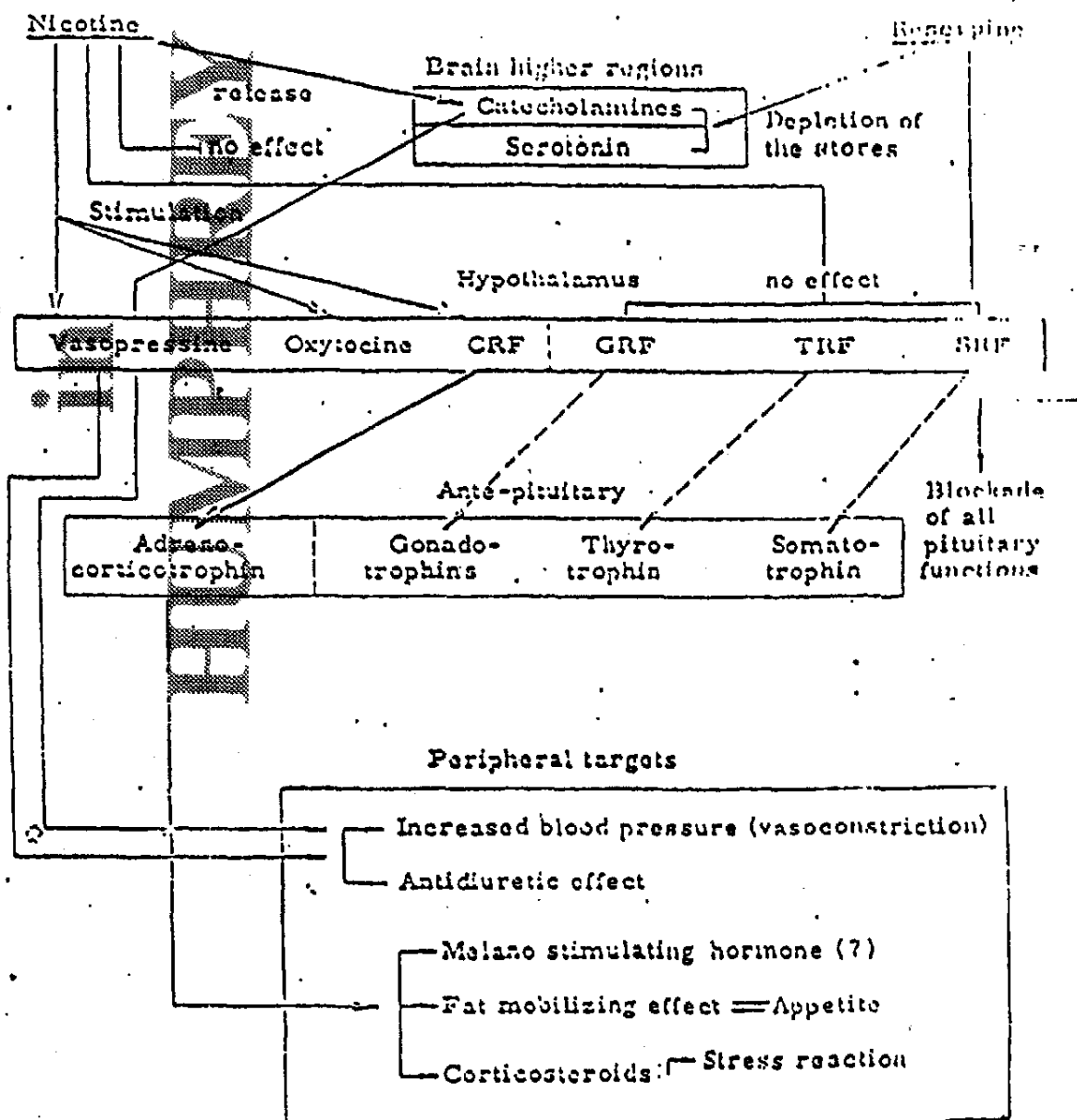
These considerations raise a very important question:

What is the reason for this specificity? Is it that nicotine acts on CRF via the release of some biochemical product from the brain?

Our attempts to explain nicotine activities on brain functions on a biochemical basis was not successful. Our working hypothesis was to study whether nicotine interferes in the brain stores of serotonin, an amine which is considered to be concerned with nervous impulses in brain. It is well known, on the one hand, that nicotine depletes the stores of catecholamines - other brain neurotransmitters - and, on the other hand, that reserpine depletes the brain stores of both groups of amines (catecholamines and serotonin). One of the hypotheses tending to explain reserpine effects on brain functions lies in this depleting activity. As an analogy, we thought it possible that nicotine might also act in a similar way. In our experiments, using a selective method for the measurement of serotonin in brain, nicotine did not act in this way. The possibility remains that other brain constituents could mediate the CRF stimulation.

A quantitative investigation of the relations with time of nicotine - and of some possible brain mediators - on adreno-corticotrophic activity could give us the key to the explanation of both phenomena of tolerance and of addiction, in showing the symptoms of withdrawal.

Diagrammatic Representation of the Effects of Nicotine and  
of Reserpine on Hypothalamo-Pituitary Functions





## I. INTRODUCTION

Jean Nicot, in his report to his Queen, Catherine de Medici, declared that smoking led to "a quiet tranquillity and great submissiveness of disposition" (1).

This sedative - or soothing - effect was a prerogative of tobacco smoking for centuries and may explain in part its extraordinary popularity.

However, the recent discovery of other "soothing" or "tranquillizing" drugs, like reserpine, a natural alkaloid from Rauwolfia, led the chemists to synthesize a number of such drugs, the demand for which increases every day. Is it possible that the use of "tranquillizers" might supersede tobacco habits? To face this possibility, it seems necessary to understand more about the physiological activities of nicotine and of reserpine that can be related to their soothing effects.

The difficulty of the research lies in the fact that nobody knows how nicotine acts on brain functions to produce its soothing effect; and nobody knows either in which way the new drugs exert their tranquillizing effects. It was necessary, then, to work on the basis of a hypothesis.

Our working hypothesis on the soothing effects of nicotine - enhancement of the normal hypothalamo-pituitary mechanism of defence against stress - was developed in the first step of this research (HIPP/O II) and led us to study the other possible actions of nicotine on the hypothalamo-pituitary system.

(1) Cited in P.S. Larson, H.B. Haag and H. Silvette: Tobacco - Williams and Wilkins, Baltimore (1961).

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A literature survey (see our First Report on HEPATO II) showed that the most generally accepted hypothesis concerning reserpine as a tranquillizer involves its depleting effect on the stores of neurohumoral agents in the brain, mainly on serotonin stores. It seems very probable that nicotine exerts its own effect on hypothalamus by way of a primary action on the biochemistry of brain which might be close to that of reserpine itself.

From another point of view, some experimental data showed that reserpine may interfere with the hypothalamic functions.

It was thus necessary to compare the effects of both nicotine and reserpine on their respective targets:

A. Comparison of the effects of nicotine and of reserpine on the hypothalamo-pituitary system;

B. Comparison of the effects of nicotine and of reserpine on brain serotonin stores.

This investigation was the object of the second step of the research (HEPATO II).

## II. COMPARISON OF THE EFFECTS OF NICOTINE AND OF RESERPINE ON THE HYPOTHALAMO-PITUITARY SYSTEM

In this part of our Report, we shall compare the experimental results obtained for nicotine in research HIRPO I \*) with investigations of similar effects of reserpine, these latter data having been gathered in the literature or resulted from our own experiments.

### A. Anti-diuretic Effect:

The anti-diuretic effect of nicotine is very well known and was studied in HIRPO I (pp. 9 - 12). Reserpine did not show any anti-diuretic activity in Burn's test at the doses of 0.25, 0.50 and 1.0 mg/kg. It was impossible to investigate the possible effects of higher doses, the treated animals having presented intestinal troubles interfering with the test. We never observed such troubles in animals receiving nicotine, even in large doses.

### B. Action of Nicotine and of Reserpine on the "Stress" Mechanism and on the Adreno-cortical Function

Three kinds of tests are available to study the adreno-cortical function and its reaction to stressful stimuli:

1. Adrenal ascorbic acid depletion test, based on the fact that the adrenal ascorbic acid concentration is depleted when the glands are stimulated, either by the pituitary adreno-corticotrophic hormone (ACTH) or in reaction to stressful stimuli.

\*) For the detailed results obtained with nicotine, please see our Final Report on Project HIRPO I.

2. Measurement of in vitro release of corticosteroids by the adrenals.

3. Measurement of in vivo concentration of plasma corticosteroids in treated animals.

All three tests were used in our experiments, to study the effects of nicotine on the adreno-cortical function.

1. Adrenal Ascorbic Acid Depletion Test.

Our results (please see Final Report on HIPRO I, pp. 13 - 18) agree quite well with those found in the literature and show that nicotine produces a marked depletion in the ascorbic acid concentration, that is to say a stimulation of the adreno-cortical function. Maren (2) found results very similar to ours and verified that nicotine does not act after hypophysectomy; its effect is therefore mediated via the pituitary.

2. Measurement of in Vitro Release of Corticosteroids by the Adrenals

The concentration of corticosteroids released by the adrenals maintained in vitro for one hour is a function of the steroidogenesis in the gland at the time of sacrifice of the animals, and therefore a parameter of pituitary adrenal activity.

The measurement of in vitro steroidogenesis was made following Saffran and Schally's technique as described by J. van der Vies (3).

- (2) T.H. Maren, Pharmacology of nicotine: antipyretic, renal and adreno-corticotrophic effects. *P.S.E.B.M.* 77, 521 (1951).
- (3) J. van der Vies, Experience with an assay of ACTH based on the steroid output of rat adrenals in vitro. *Acta Physiol. Pharmacol. Neerland.* 5, 361 - 384 (1957).

Method. The method was followed exactly as described by J. van der Vies:

Performance of the Test

From stock solutions, using analytical reagents and water, double-distilled in an all-glass apparatus, the following volumes are mixed:

4.50 % (w/v)	sodium chloride	200 ml
5.75 %	potassium chloride	8 ml
6.10 %	calcium chloride	6 ml
10.55 %	monopotassium phosphate	2 ml
19.10 %	magnesium sulphate	2 ml
	water up to	545 ml

~~Never.~~ The calcium chloride should be added after the mixture of the other constituents has been diluted to approximately 500 ml with distilled water. A precipitate may otherwise occur which makes the solution unsuitable.

This mixture, indicated hereafter as "double Ringer", is usually stable in the refrigerator for a fortnight. As soon as the solution deteriorates by the appearance of moulds or a precipitate, it should be discarded. The same holds for the stock solutions which are also kept in the refrigerator.

Shortly before use, the final medium is prepared as follows:

- To 27 ml of double Ringer is added:
- 13 ml of 1 % glucose in water (freshly prepared each day)
- 14.5 ml of water
- 10.5 ml of 1.30 %  $\text{NaHCO}_3$  which has been gassed with carbon-dioxide at room-temperature for one hour. The pH of this solution should be 7.4.

The final Ringer solution is gassed with a mixture of 95 %  $O_2$  and 5 %  $CO_2$  for 10 minutes.

Eight young male rats are used for each assay. Although rats having bodyweights of 135 - 200 g may be used, the weight of the rats for a single test should be within a range of about 20 g.

The animals are anaesthetized in the animal house by an intra-peritoneal injection of 5 mg Nembutal per 100 g bodyweight. The Nembutal used is a commercial preparation (Abbott Laboratories, London) which is dissolved in saline to the appropriate concentration shortly before use.

Rats which are disturbed, either by weighing or by injection, should not be used. It proved essential for the handling of the rats before anaesthesia developed to be done solely by the people normally occupied in the animal house.

The rats are brought into the laboratory when anaesthesia is complete. The adrenals are removed from each rat and put into a petri dish lined with moistened filter-paper, after which the glands are free from adhering tissue by means of a small lancet, taking care that the adrenals are not damaged. Each adrenal is carefully cut into quarters with fine, sharp scissors. The quartering should be as even as possible; after some practice, eight nearly equal quarters can be cut from a pair of adrenals. The quarters are then placed into eight numbered sectors of a filter-paper circle, moistened with medium and kept in a petri dish. The adrenals of the eight rats are thus cleaned, quartered and distributed into eight lots in the petri dish, each sector containing eight quarters of adrenals from all eight rats. The adrenal quarters in each sector are weighed on a micro-torsion balance (maximum capacity 50 mg) and then placed in Warburg flasks containing 1.5 ml of medium.

The flasks are mounted on Warburg manometers, well-flushed with a mixture of 5 %  $\text{CO}_2$  - 95 %  $\text{O}_2$  and shaken in the bath at  $38^\circ\text{C}$  for one hour and a half.

The flasks are removed from the bath at the end of the incubation period. Aliquots of the medium of one ml each are transferred from each flask to tubes containing 2 ml of methylene chloride. The tubes are then well stoppered and manually shaken for one minute. The contents generally emulsify. The stoppered tubes are centrifuged at about 2 000 r.p.m. to separate the two phases. A part of the lower phase is transferred with a long needle and syringe to a quartz microcuvette, and readings are taken at 240 and 255 m $\mu$  in a Beckman model D.U. ultra-violet spectrophotometer. The blank cuvette contains pure methylene chloride. The individual absorption of each cuvette, filled with pure methylene chloride, is determined before each series of readings. Differences in absorption between the cuvettes are taken into account when the extracts are read. The lamp housing of the spectrophotometer must be cooled to avoid evaporation of the solvent. During the readings, the tubes awaiting attention must be kept stoppered.

#### Purification of Methylene Chloride

The reagent grade methylene chloride is washed with an equal volume of water, dried with anhydrous sodium sulphate and decolourized with charcoal. The solvent is kept over sodium hydroxide flasks for about 24 hours and is then distilled; the fraction boiling between  $40^\circ$  and  $41^\circ\text{C}$  is collected.

The assay of pure corticosterone was fairly reproducible and gave a good curve (difference between optical densities at 240 m $\mu$  and 250 m $\mu$ ; Beckman D.U. apparatus). Of the quantity of corticosterone added to the medium, 85.5 % to 105.8 %, was recuperated.

### Results

This method was used in two cases:

- (a) In tolerant rats and their controls; the adrenal weight and the corticosteroid release by the glands may give information on a possible effect of prolonged nicotine treatment on the hypothalamo-pituitary adrenal system.
- (b) In fresh rats submitted to a surgical stress and receiving acute nicotine administration.

#### (a) Tolerant Rats

Data are gathered in Table I.

From these results we can make the following observations:

- (i) Prolonged treatment by nicotine giving way to the development of tolerance increases the weight of the adrenals; this adrenal hypertrophy is a sign of corticotrophin hyperactivity.
- (ii) The total amount of corticosteroids released in 1 1/2 h by one adrenal is increased in tolerant rats vs their saline controls. This same amount, calculated per 100 g body weight, is also increased, while the calculation per 100-mg adrenal does not show any significant difference between the two groups of animals. This fact indicates that the total increase in corticosteroid production is due to adrenal hypertrophy, and not to an increased production by every cell in the adrenal.
- (iii) It thus seems that total corticosteroid production is actually increased in tolerant rats.



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Table 1

Effect of Prolonged Nicotine Treatment on the Adreno-cortical Function

Adrenal weight		Adrenal corticosteroids					
mg/100 g body weight		$\gamma$ /adrenal		$\gamma$ /100 mg adrenal		$\gamma$ /100 g body weight	
Saline controls	Tolerant rats	Saline controls	Tolerant rats	Saline controls	Tolerant rats	Saline controls	Tolerant rats
10.9	11.8	2.75	3.75	12.3	13.9	1.69	2.24
11.1	13.4	3.75	4.00	14.3	17.3	1.94	2.33
12.3	14.0	3.90	5.00	17.8	19.2	2.21	3.00
12.4	14.6	4.25	5.15	19.8	19.2	2.34	3.11
12.6	14.8	4.25	5.35	20.8	20.0	2.37	3.12
13.2	14.9	4.35	5.95	20.8	20.6	2.53	3.16
13.2	15.1	5.00	6.10	20.9	21.3	2.59	3.31
13.6	15.2	5.15	6.15	21.2	23.2	2.61	3.37
15.2	15.5	5.65	6.65	23.7	24.9	3.56	3.45
	15.6		6.65		25.2		3.52
	16.2		6.75		26.3		3.89
	16.2		7.05		27.2		4.05
	16.5		8.70		28.5		4.34
	16.9		8.70		32.7		4.55

It will be necessary to investigate the possibility of an increase in the amount of corticosteroids in the blood of smokers.

(b) Fresh Rats Submitted to Surgical Stress

We first verified that a surgical stress increases the amount of corticosteroids released by the adrenals in vitro.

The left adrenal was removed within one or two minutes after the rat had been anesthetized in the animal house; the right gland was taken out 15 minutes after the removal of the left one; the animal woke up in the meantime. Two adrenals of one side were put together into a Warburg flask.

The difference between the amount of steroids released by the right adrenals (surgical stress) and the left ones (rest state) was significant, as can be seen from our data (Table 2):

Table 2

Action of a Surgical Stress on Release of Corticosteroids in Vitro

Corticosteroids in  $\mu$ g per 100 mg adrenal

Rat No	Left adrenal	Right adrenal	$\Delta$ % left adrenal
1 + 2	17.4	24.5	+40.8
3 + 4	15.6	26.0	+66.7
5 + 6	13.6	25.2	+85.3
7 + 8	12.7	18.5	+45.2
9 + 10	19.7	26.0	+33.0
11 + 12	23.9	29.5	+23.5
13 + 14	21.5	39.7	+85.1
15 + 16	22.8	42.1	+85.0

These data confirm that the technique is suitable for measuring in vivo ACPH release, as was stated by the authors.

#### Action of Nicotine

Twenty-two rats were used in two groups (12 control rats and 10 rats treated with 4 mg/kg nicotine).

Our schedule was the following:

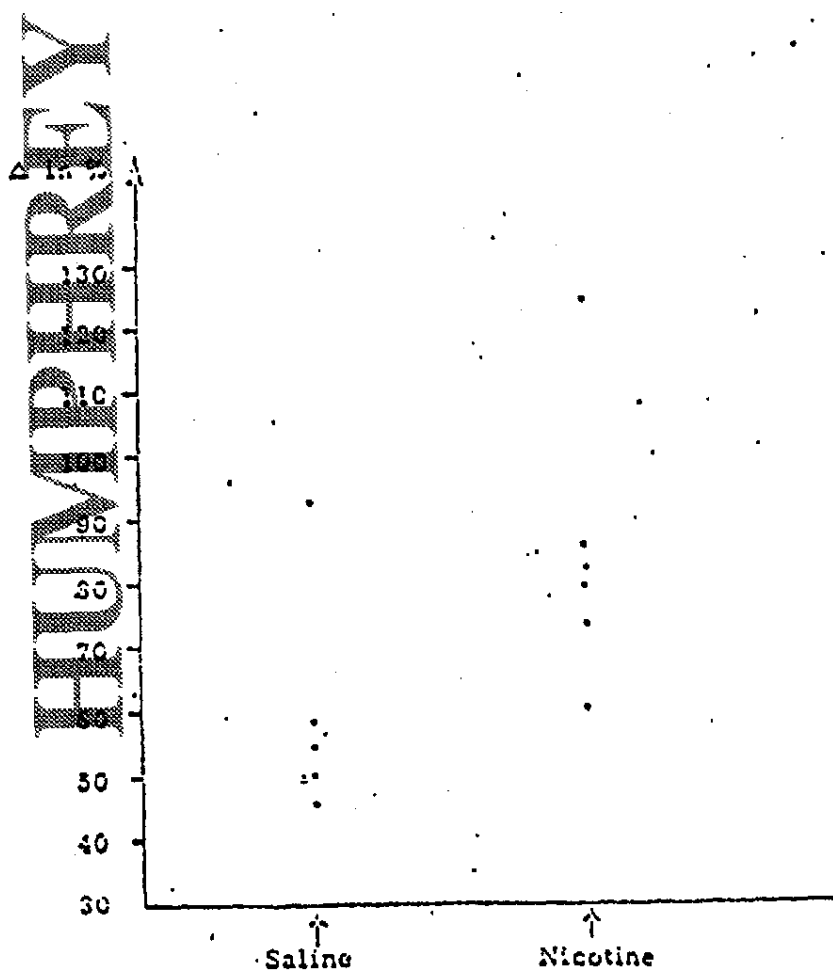
- Time 0 : anaesthesia of the rats with Nembutal.  
20 min. : removal of the left adrenal of every rat, followed immediately by injection of saline (control group) or nicotine.  
The left adrenals of two rats are put together in each Warburg flask.  
35 min. : removal of the right adrenal of every rat; two right adrenals are put together in each Warburg flask.

All the flasks are put in a water bath ( $37.5^{\circ}\text{C}$ ) and shaken for 90 minutes. After this time an aliquot of the medium is taken out from every flask, extracted by methylenechloride, and the corticosterone is measured in methylenechloride with the Beckman D.U. apparatus.

Data are shown in Fig. 1.

Every point represents the difference between the corticosteroids released by two right adrenals and the corticosteroids released by the two left adrenals of the same rats.

Fig. 1 Action of nicotine on corticosteroids release



This figure shows a definite increase in this difference when the rats are treated with nicotine. This fact clearly indicates that nicotine enhances the normal reaction to stress, and is in agreement with the results of the AAAA test.

Attempts to investigate the time relationship between nicotine administration and the adrenal corticosteroid production showed that the "in vitro" technique is not really suitable for this investigation: results were not sufficiently reproducible, and it was not possible to make safe conclusions from them.

It seemed therefore necessary to measure the plasma corticosteroid concentration.

### 3. Measurement of Corticosteroid Concentration in Plasma

The only technique available for the time being to measure plasma corticosteroid concentration in rats is that of Silber et al. (4), generally used following the method described by Guillemain et al. (5). A thorough investigation of this method led to the conclusion that it measures mainly, out of the corticosterone itself, the circulating lipids and free fatty acids. As we had previously shown (see Report HYPVO I, Fig. 16) that nicotine releases very rapidly the free fatty acids in the blood, it was senseless to use this technique to investigate a specific activity of nicotine on blood corticosterone itself. Such a determination would need a thorough investigation of an entirely new technique.

It thus seemed necessary to limit the present research to the above data.

- 
- (4) R.H. Silber, R.D. Busch and R. Oslapas, Clin. Chem. 4, 278 (1958).  
(5) R. Guillemain, G.W. Clayton, J.D. Smith and H.S. Lipscomb, Endocrinology 62, 349 (1956).

#### 4. Action of Reserpine on the Pituitary-Adrenal Response to Stress

Guillemin (6) showed that the tranquilizers chlorpromazine and reserpine can deplete adrenal ascorbic acid when administered to rats for a short time (less than 4 days). However, this enhanced release of ACTH does not last longer, and the results demonstrated the existence of an "adreno-corticotrophic adaptation" to these drugs. Most authors think that prolonged reserpine treatments lead to corticotrophic exhaustion.

Nicotine does not seem to produce such a phenomenon, as we observed a marked adreno-corticotrophic effect on the weight of the adrenals of tolerant rats, and on their total corticosterone production as well after a six-week treatment by nicotine (see Table 1).

Furthermore, nicotine enhances the normal response of our rats to a stressful situation (surgical procedure) as can be seen in Fig. 1, while Guillemin (6) saw no enhancement of the response to stress in the A.A.D. test neither by reserpine nor by chlorpromazine.

#### C. Action on Body Weight Regulation

The many effects of nicotine on body weight regulation were studied in HIPRO I (pp. 22 - 33).

We did not find any indication in the literature concerning such effects of reserpine. However, reserpine decreased very slightly the appetite in our "appetite test" on rats, as can be seen in Fig. 2. The response does not seem to be proportional to the log-dose. (Compare with the response to nicotine, Final Report on HIPRO I, Fig. 7.)

(6) R. Guillemin, in W.S. Fields: Brain mechanisms and drug actions, 99 - 110 (1957).

#### D. Action on Other Hypothalamo-pituitary Functions

##### 1. Possible Effects on the Pituitary Thyrotrophic Function

Experimental data of HIPPO I (pp. 38 - 42) showed that nicotine did not seem to interfere with the regulation of pituitary thyrotrophin function by the hypothalamus. Data found in the literature are very variable but seem to show that nicotine may have some slight stimulating effect on thyroid function (cf. Larson et al., (1), pp. 368 and 373 - 375). As regards reserpine activity, the effect on the contrary would be an inhibiting one (7).

##### 2. Effects of the Pituitary Gonadotrophic Function

###### (a) Female

Experimental data of HIPPO I showed that it was necessary continuously to increase the daily dose of nicotine in the course of a chronic treatment until 16 mg/kg/day to obtain a blockade of both gonadotrophins and an absence of oestrous cycles in some of the treated rats (Final Report on HIPPO I, pp. 42 - 43). Data found in the literature generally agree with these results (see Larson et al. (1), Ch. 12).

Reserpine seems to be much more potent than nicotine as regards "anti-gonadotrophic" functions.

We investigated this effect in the following way:

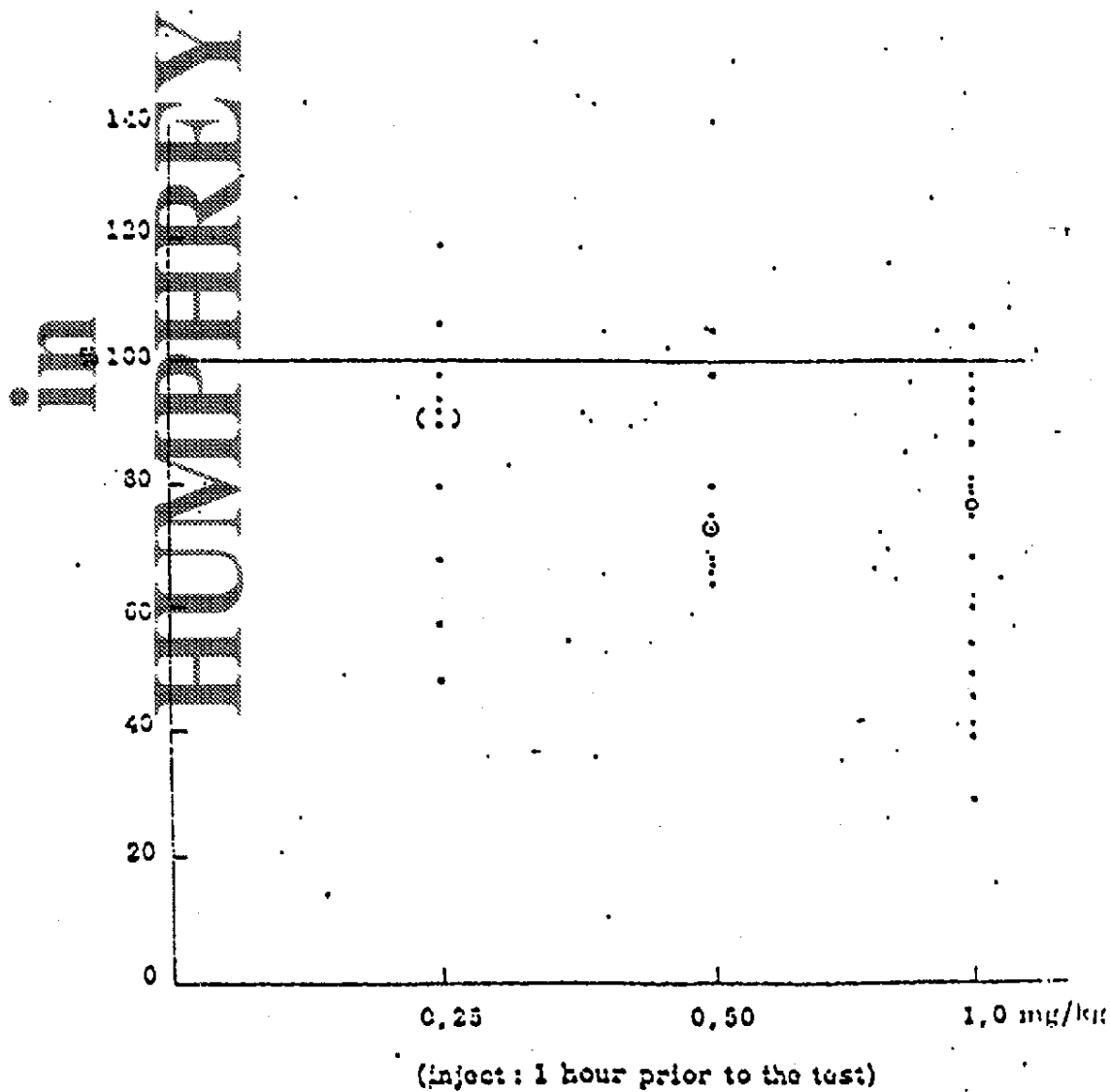
- (7) G. Darnaud, Y. Denard, G. Moreau, R. Volsin et Y. Calchies: Action de la réserpine sur l'hyperthyroïdie, *Pr. Méd.* 57 (12), 457 - 460 (1959).

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Fig. 2

Response to pentobarbital in the narcosis test

(1 hour feed intake % of control day)





Five normal female rats having very regular four-day cycles received one single injection of reserpine (1 mg/kg) on the day of pro-oestrus. This unique dose of reserpine was sufficient to block completely both gonadotrophic functions and oestrous cycles in four of the five rats treated. In one rat, the blockade lasted for three weeks, in two rats for two weeks, and in one rat for one week following the unique dose of reserpine.

The oestrous index calculated for the five treated rats was 0.000 during the three weeks following the injection, instead of 0.220 during the two-week control period prior to the injection of reserpine (compare with Table 9, p. 43 of Final Report on HLP-O I).

Our data agree quite well with others found in the literature: Barraclough and Sawyer (3) found that reserpine (1 mg/kg) blocked oestrous in 90 % of the rats treated. In prolonged treatment, doses as small as 100  $\mu$ g/kg/day for 1 to 3 weeks were sufficient to interfere with the female cycle (9). Khazan et al. showed that "reserpine at a dose level of 0.2 mg/kg delayed vaginal opening in infantile female rats, and postponed oestrous in normally cycling female rats. In women, vaginal smears likewise indicated decreased oestrogen production throughout treatment" (10).

#### (b) Male

In the male, Verne et al. (11) and Soulaïrac (12) found an inhibition of the testicular endocrine function, in response to reserpine

- (3) C.A. Barraclough and C.H. Sawyer: Blockade of the release of pituitary gonadotrophins in the rat by chlorpromazine and reserpine. *Endocrinol.* 51, 341 (1957).
- (9) R. Gaunt et al.: Endocrine aspects of the pharmacology of reserpine. *Ann. N.Y. Acad. Sc.* 59, 22 - 35 (1954).
- (10) N. Khazan, F.G. Sulman and H.Z. Winnik, Effect of reserpine on pituitary-gonadal axis, *P.S.E.B.M.* 105, 201 (1960).
- (11) J. Verne, H. Tuckmann-Duplessis et S. Hebert. Etude comparative de l'influence exercée par la reserpine et par l'hypophysectomie sur le testicule et la vésicule séminale du rat, *Ann. d'Endocrinol.* 19 (6), 952 (1958).
- (12) J. Soulaïrac, *ibid.* 19 (6), 953 (1958).

(500  $\gamma$ /kg/day). The inhibiting effect was much more pronounced than the light one that we found in our seven-month nicotine-treated rats (Final Report on HIRPO I, pp. 44 - 45).

It appears, therefore, that reserpine inhibits gonadotrophin function much more markedly than nicotine.

It seems very likely that the effects of heavy daily doses of nicotine on the oestrous cycle are mainly due to the malnutrition consecutive to the inhibiting effect of nicotine on appetite.

On the contrary, a unique small dose of reserpine must act directly on the gonadotrophic regulation system.

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### III. COMPARISON OF THE EFFECTS OF NICOTINE AND OF RESERPINE ON BRAIN SEROTONIN STORES

#### A. Literature Data

##### 1. Reserpine Effects on Brain Amine Stores (Please see First Report on HLP-011, pp. 5 - 9)

Data in the literature show that the brain stores in catecholamines (adrenaline and noradrenaline) as well as in serotonin (5-hydroxytryptamine or 5-HT) are depressed after reserpine administration to subjects. Serotonin urinary metabolite, 5-hydroxy indole acetic acid (5-HIAA), is correlatively increased in urine after reserpine administration.

As an attempt to summarize data concerning the biochemical effects of tranquillizing drugs on the brain stores of the neurohormones, we were led to make the following observations:

- (a) Serotonin and the catecholamines seem to inhibit the transmission of the nerve impulse in the brain synapse, acetylcholine on the contrary being the mediator of the transmission.
- (b) The tranquillizing effects of reserpine could be due partly to the observed depletion in the inhibiting amines, thus leaving the acetylcholine activity unopposed. Some of the pharmacological effects observed after the administration of reserpine are actually signs of acetylcholine activity.
- (c) From another point of view, the depletion of the amines from the body stores deprives the organism of part of the mechanism of reactivity, thus decreasing the manifestations of anxiety and tension.

## 2. Nicotine Effects on Brain Amine Stores

It was shown that nicotine releases nor-adrenaline from its stores, and Prof. J.H. Burn thinks that "the pleasure of smoking is derived in part from the release of nor-epinephrine from its stores in the brain by nicotine, the release giving an increased feeling of cheerfulness and a sense of relief from fatigue"(13).

In 1961, Werle (14) showed, that nicotine (0.31 to 0.50 mg/kg) depletes the brain serotonin stores to 50 % in the dog in less than 30 minutes.

### B. Experimental Investigation

We investigated the effects both of reserpine and of nicotine on the brain serotonin stores, and the effects of nicotine on the elimination of the metabolite 5 HIAA.

#### 1. Methods

##### (a) Selective Determination of Serotonin in Brain

We chose to follow the method of Bogdanski, Pletscher, Brodie and Udenfriend (15) which consists in the measurement of the fluor-

- 
- (13) J.H. Burn, The Action of nicotine on the peripheral circulation. Annals N.Y. Acad. Sc. 90 (1), 81 - 84 (1960).
- (14) H. Schlievelbein, E. Werle and W. Jacoby. Ueber die Freisetzung von 5-Hydroxytryptamin (Serotonin) aus Geweben durch Nikotin. Die Naturwissenschaft 40 (10), 602 (1961).
- (15) D.F. Bogdanski, A. Pletscher, B.B. Brodie and S. Udenfriend. Identification and assay of serotonin in brain, J. Pharmacol. exptl. Therap. 117, 82 (1956).

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escence developed by serotonin in 0.1 N HCl at 530 m $\mu$  resulting from activation at 295 m $\mu$  (AMINCO-BROWMAN spectrofluorometer). It had formerly been shown that measurement of fluorescence at 530 m $\mu$  was not specific for serotonin, and therefore not suitable for its determination in brain (16).

The details of the method were as follows:

#### Solvents

The solvents were purified before use in the following way:

n-Butanol and n-Hexane - Successive washing with N NaOH, N HCl and distilled water until pH 7.0; distillation of the washed solvent.

Borate buffer - 32.8 g boric acid was dissolved in an aqueous solution of NaOH (110 ml of 10 N NaOH in 2 litres of distilled water). The solution was saturated with 200 ml n-butanol and then with NaCl. The butanolic phase was eliminated. The buffer must be at pH 10.0.

#### Procedure

One part of brain tissue was homogenized with two parts of 0.1 N HCl in a Potter tube. 9 ml of the homogenate was transferred in each of 60-ml glass-stoppered flasks and adjusted to approximately pH 10 by the addition of anhydrous sodium carbonate; 5 ml of borate buffer pH 10 was added, with 1 ml of distilled water (final volume = 15 ml). 5 g NaCl and 20 ml of n-butanol were added, and the mixture was shaken for 15 minutes and then centrifuged. The butanolic phase was removed by aspiration and put in a new 60-ml flask with a 10-ml borate buffer for washing. (The butanolic phase was washed twice, and centrifuged.)

(16) S. Udenfriend, D.F. Dogliani and H. Weissbach, Fluorescence characteristics of 5-HT (serotonin), *Science* **122** (No 3177), 1072 (1953).

15 ml of the washed butanolic phase was removed and put in a new 60-ml vessel with 50 ml of n-heptane and 5 ml of 0.1 N HCl. The mixture was shaken for 1 minute and centrifuged, then the underlying aqueous layer was removed for determination of fluorescence after adding 0.6 ml of concentrated HCl to 2 ml of the 0.1 N HCl solution (3N HCl).

The solution is activated in the Aminco-Fluorometer apparatus at 295 m $\mu$  and the resultant fluorescence is measured at 550 m $\mu$ .

(b) Colorimetric Determination of 5-hydroxy-3-indole Acetic Acid in Urine

We followed the method described by Udenfriend et al. (17) as follows:

"To 6 ml of urine in a 50-ml glass-stoppered bottle are added 6 ml of 2,4-dinitrophenylhydrazine reagent (0.5 % 2,4-dinitrophenylhydrazine in 2N HCl). After 30 minutes, 25 ml of CHCl<sub>3</sub> are added, and the bottle is shaken for a few minutes and then centrifuged."

"The organic layer is removed, placed with a fresh 25-ml portion of CHCl<sub>3</sub> and the extraction repeated. After centrifuging, a 10-ml aliquot of the aqueous layer is transferred to a 40-ml glass-stoppered centrifuge tube containing about 4 mg of NaCl and 25 ml of ether. The tube is shaken for 5 minutes. Following centrifugation a 20-ml aliquot of the ether is transferred to another 40-ml glass-stoppered centrifuge tube containing 1.5 ml of buffer at pH 7.0. The tube is shaken for 5 minutes, centrifuged, and the ether layer is removed by aspiration. 1 ml of the aqueous phase is transferred to a 15-ml glass-stoppered centrifuge tube containing 0.5 ml of nitroscaphitol reagent (0.1 % solution in ethanol)."

(17) S. Udenfriend, E. Titus and H. Weisbach, The Identification of

" Following this, 0.3 ml of nitrous acid reagent is added (to 5 ml of 2 N  $H_2SO_4$  is added 0.2 ml of 2.5 %  $NaNO_2$ ; the reagent should be prepared freshly daily) and the sample is mixed well and warmed at  $37^\circ C$  for 5 minutes; 5 ml of ethyl acetate are then added and the tube is shaken."

" After separation of the phases and removal of the ethyl acetate by aspiration, a second 5-ml portion of ethyl acetate is added, and this step is repeated."

" The final aqueous layer is transferred to a micro cuvette, and the optical density is measured at 540 m  $\mu$ ."

" Standards are prepared by treating 6 ml of solution containing 10 to 100  $\gamma$  of 5 LAA exactly as for the urine samples. The reagent blank used for the blank setting of the instrument is prepared by treating 6 ml of water in the same manner."

## 2. Results

### (a) Effect of Reserpine on Brain Serotonin Stores in Rats

Fig. 3 shows the results of our investigation of the rat brain serotonin content, the animals being sacrificed 15 hours after the administration of various doses of reserpine (0.3, 1.0, 3.0, 10.0 mg/kg). Each point represents the pool of three brains.

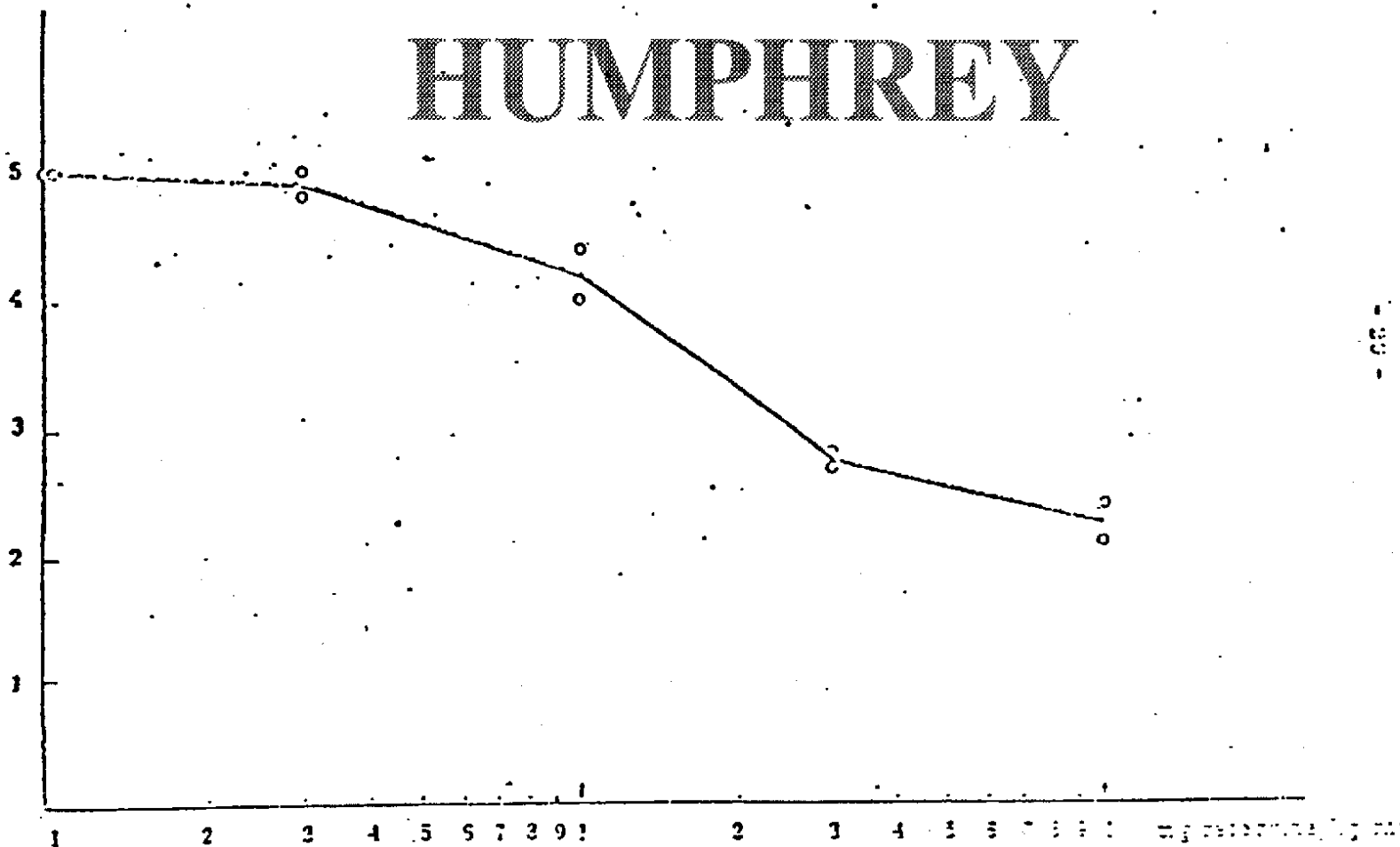
In our hands the depletion of serotonin brain stores was not to be found for doses below 1.0 mg/kg; the effect was approximately proportional to the log dose from 1.0 to 10.0 mg/kg.

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Fig. 3

Brain Serotonin after Reserpine Treatment

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(b) Effect of Nicotine on Brain Serotonin Stores in Rats

Fig. 4 shows that, under our experimental conditions, nicotine does not seem to have any depleting effect on rats brain serotonin stores for the dose of 1 mg/kg during the first two hours after administration of the drug.

(c) Effect of Nicotine on 5 HIAA Eliminated in Urine

Table 3 shows the elimination of the serotonin metabolite in the urine of rats having received nicotine (4 mg/kg subcutaneously) or saline, the urine being collected for the 6 hours following the injection.

Table 3

Effect of Nicotine (4 mg/kg) on 5 HIAA Elimination in Urine

Urinary concentration γ/10 ml		Urinary excretion γ/24 h	
Saline	Nicotine	Saline	Nicotine
10.3	5.3	35.4	17.4
11.7	6.7	20.4	20.5
13.1	9.5	32.6	34.5
13.6	9.7	35.3	35.1
13.1	11.7	36.1	36.4
17.2			

Table 4 shows the urinary concentration and the urinary excretion of serotonin in tolerant rats, in resistant rats and in their controls.

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Fig. 4

Brain Serotonin after Nicotine Treatment

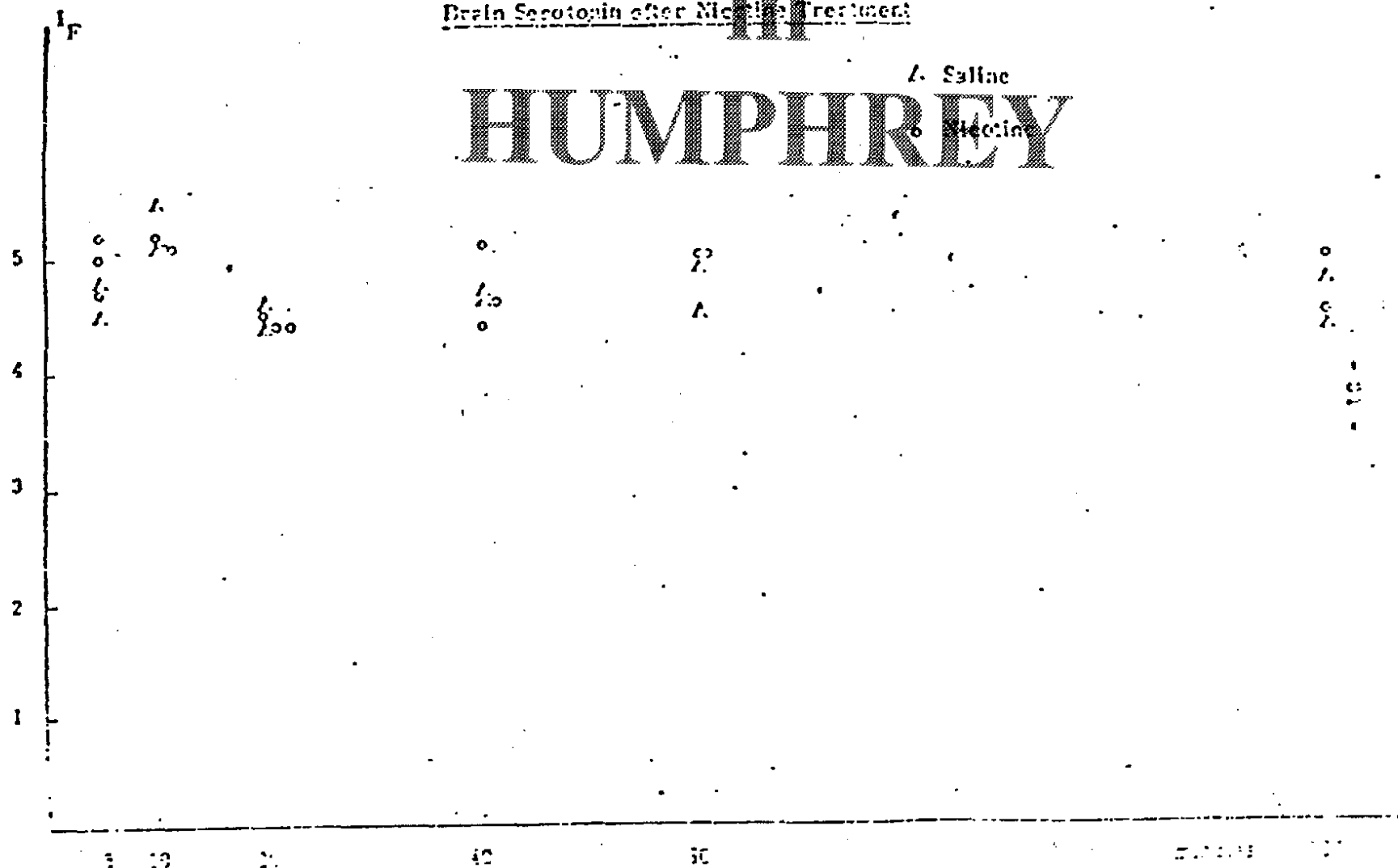


Table 4

Urinary concentration of 5HIAA ( $\gamma$ /10 ml)		Urinary excretion of 5HIAA ( $\gamma$ /24 h)	
Control rats (NaCl 0.5%)	Treated rats (Nicotine 2x2 mg/kg)	Control rats (NaCl 0.5%)	Treated rats (Nicotine 2x2 mg/kg)
4.50	5.60	7.95	9.45
5.94	5.64	9.45	10.62
6.27	6.50 (R)	10.20	11.75
7.10	7.50	10.60	12.30
7.10	7.52	10.80	12.15
7.50	7.52	10.80	12.10
7.51	7.91	11.50	12.50 (R)
7.52	8.75	12.40	13.35
8.75	8.75	12.50	14.30
8.75	9.33 (R)	12.71	14.86
8.80	9.33	13.30	15.01
9.60	9.33	13.35	15.50
9.60	9.60	14.30	15.70
9.60	9.60	15.40	16.25
9.60	9.60	15.50	16.60
9.61	9.61	17.30	17.05
9.61	10.00	17.50	17.40
9.80	10.00	17.60	17.55
10.40	10.12	17.60	17.56
10.60	10.12	18.60	18.85
11.25	10.40	19.40	19.00
11.25	10.60	19.60	19.30
11.65	10.40	19.55	19.30
12.20	11.60	20.85	19.60
13.35	11.23	21.20	19.70
13.55	11.25	21.30	19.90 (R)
14.05	11.65	22.05	20.90
14.52	11.33	23.60	21.10
15.40	12.05	24.60	21.90
15.80	12.05 (R)	26.00	21.95 (R)
21.20	12.10	27.00	23.70
	12.10	31.50	23.90
	15.35		24.30
	17.20		24.60
	19.75		25.75
	20.60		28.40
	21.70		30.00

From our results it seems that nicotine does not act in depleting serotonin stores from brain or other parts of the body.

Its action on brain seems therefore entirely different in this respect from that of the tranquilizer reserpine.

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Geneva, April 30, 1963  
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## Composition of Papers on U.K. Manufactured Cigarettes

<u>Type of Paper</u>	<u>Share of Trade</u>	<u>Loading</u> (Chalk of 90.5% purity)	<u>Furnish</u> (Pure Cellulose)
A	)	25	75
B	)	25	75
C	) Over 90%	25	75
D	)	25	75
E	)	25	75
F	)	25	75
G	Very small	22½	77½
H	" "	20	80
I	" "	15	85
J	" "	15	85
K	" "	15	85
L	" "	15	85
M	" "	12	88
N	" "	3	97

75.25/4.

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APPENDIX 12 REPORTS ON PROJECT HIPPO 1 & 2  
AND PROJECT MARY 1 & 2 & 3

General Comments

These reports represent a preliminary survey of some actions of nicotine, which it was considered might be related to the cigarette smoking habit. The data in these reports have been poorly presented by any standards and even for the non-critical and non-technical reader there is so little detail that the experiments are in some cases impossible to follow. One of us (A.K.A.) has been to the Battelle Institute, Geneva, and obtained certain information that was either missing or not clear in the reports. Dr. Liber has been responsible for most of the Hippo work and in fairness it should be said that Dr. Liber is an endocrinologist by training and not a pharmacologist or biochemist. (The Pharmacology Division of Battelle was fully occupied doing chronic toxicity work for the Swiss Pharmaceutical Companies). Dr. Kasalbach is a Biochemist.

It is almost universal in making a report about work on rats to state the kind of rats used, whether the rats are highly inbred or rats of mixed strain, whether they are for example, Wistar, Sprague Dawley, hooded or black and white rats. This information gives an indication of the variation to be expected since highly inbred rats are much more uniform in behaviour. The rats used by Battelle were white rats claimed to be of the Wistar strain and were obtained from a local dealer. It is also standard practice to use what are called "litter-mate controls" in all cooperative experiments of the type undertaken by the Battelle workers. Thus in three groups of rats, one being a control group and the other two being groups in which an agent such as nicotine is given, each rat in one group has a brother or sister rat in the other groups. Battelle were apparently unable to obtain litter-mate rats so that the data of the Hippo reports are no more than qualitative. Rats should have been obtained from a breeder rather than a dealer; any reputable breeder will always supply litter-mate rats on request. In addition to these criticisms of the actual animals used in the experiments, A.K.A. was not impressed with the animal accommodation at Battelle, and for long term chronic experiments it is important that the animals should be well cared for.

Solutions of nicotine were appropriately diluted and adjusted to pH 7 in all experiments. Nicotine acid tartrate is normally used in this country and this might account for some discrepancies.

PROJECT HIPPO 1

ACTION OF NICOTINE ON THE MECHANISM OF DRUGS (Section II.2.2 et seq.)

Observations were made on "fresh," "tolerant" and "resistant" rats. Tolerant rats should be able to withstand a dose of nicotine that would be lethal to fresh rats (p.8) and a measurement of the LD50 of nicotine in fresh rats and tolerant rats would give a perfectly reasonable measurement

of tolerance. Tolerance, however, was induced and assessed by a method described by Behrend and Thienes (*J. Pharmacol.*, 23, 327, 1955) in which a non-specific pivoting response of nicotine is measured. We do not think this is a good method although Dr. Libert has shown us some data indicating that this response is absent in "tolerant" rats. We think the response could have been due to irritation caused by injection of the base, which might have resulted in a progressively smaller response. Dr. Libert denied this and says there was no sign of swelling or fibrous tissue on a macroscopic examination at autopsy.

#### Experiments illustrated in Fig. 1

No control is shown for the excretion of water by rats not given nicotine. This omission makes it impossible to judge the meaning of the lines in Fig. 1. The rats were given water and nicotine by mouth at zero time and the nicotine should have delayed its excretion. The authors say that the effect of nicotine was exerted from 2-8 hours after zero. The action of nicotine, however, is observed after 15 minutes, and the dose which was used would have finished its action by 3 hours, or at the most 4 hours. The differences between the controls, the resistant rats and the tolerant rats have no significance in view of the absence of a control without nicotine and in view of the delay before the alleged nicotine effect appeared. Dr. Libert was herself puzzled by this result.

#### Possible action of nicotine via the release of noradrenaline (Section ID.2.10)

It is certainly likely that the action of nicotine in causing an antidiuretic action has nothing to do with release of noradrenaline. Such an idea has never been suggested. However, the results in Fig. 3 showing the effect of nicotine on rats given reserpine merely express the result as a plot of dose against time in which 50% of the cumulative amount of urine was excreted and there are no observations showing the time course of the excretion of water alone. It may be that there is a difference in rate of excretion of rats given reserpine alone, and those given nicotine after reserpine, but on this no information is available.

#### Influence of hypothalamic lesions (Section ID.3.11)

From the results of this section the authors conclude that "nicotine does not seem to stimulate the hypothalamic centre (supraoptic nucleus) that synthesises the antidiuretic hormone". It can be said with reasonable confidence that this conclusion is wrong. McEwen demonstrated that the anti-diuretic effect of acetylcholine was exerted only when an injection was made into the supraoptic nucleus and not when the injection was 0.5cm outside. Nicotine acts in the same way as acetylcholine and therefore it should be without action when the supraoptic nucleus is destroyed. The destruction of the nucleus by electrocoagulation using a stereotactic

apparatus is a highly skilled procedure, and the most likely conclusion is that the authors were unable to obtain a sufficiently precise localization and therefore did not in fact destroy the supraspinal nucleus. Dr. Libet agrees with this criticism and it is extraordinary that a scientist can report on a series of experiments and when questioned about their validity admit that they were not properly carried out.

ACTION OF NICOTINE ON THE "STRESS" MECHANISM (Section III, p.12 et seq.)

The experiments in this section involve biochemical as distinct from pharmacological and surgical methods and are therefore less open to errors of technique.

Action of nicotine in the adrenal ascorbic acid depletion test (Section IIIA, p.13)

The figures on p.14 (Table 1) for control rats agree with figures published by others and it is likely that the effect of nicotine is to reduce the adrenal ascorbic content as they say. The details of these experiments are now available and it is intended to repeat these crucial observations. Professor Buttle has made observations on the action of nicotine on adrenal weight and so far has failed to observe an effect. The Battelle workers have only found an effect on adrenal weight when nicotine was administered over prolonged periods.

Action of nicotine in the adrenal ascorbic acid depletion test after reserpine treatment (Table 2, p.15)

The observations of the diminished effect of nicotine after reserpine treatment are certainly interesting and worth further investigation, because the depleting action of nicotine may involve the release of adrenaline from the adrenal medulla. Adrenaline itself depletes ascorbic acid in the adrenal glands.

Action of nicotine in the adrenal ascorbic acid depletion test after morphine treatment (Table 3, p.17)

The evidence here does not justify the conclusion that the depletion of ascorbic acid by nicotine after giving morphine was less than without morphine (though it may in fact be so) because there were no rats given nicotine only in the experiments of Table 3. It is not permissible to assume that the figures of Table 1 (p.14) are comparable.



Section 10.12.1

These results involve making lesions by electro-coagulation in the median eminence of the hypothalamus. In view of the apparent failure of the authors to destroy the supraoptic nucleus by electrocoagulation, we think that these results should be treated with reserve. However, since the results agree with the data of the adrenal ascorbic acid depletion test they may be correct.

ACTION OF NICOTINE ON BODY-WEIGHT REGULATION (Section 10.12.2 (a) sec.)

Fig. 6

Litter-mate rats were not used for the experiments illustrated in Fig. 6 for the increase in weight of rats dosed chronically with nicotine and the data are therefore of doubtful significance.

Measurement of anorexic activity (Section 10.12.2 (b) sec.)

We are not familiar with the method used but it would appear to be a bad method since rats normally eat at night and they do not like a powdered diet. The Battelle rats, however, as A.R.A. was informed, flourished on a powdered diet. Appetite was measured for only one hour within an hour of dosing with nicotine and it is likely that any drugs would show transient anorexic activity under these conditions. The dose of nicotine (2 mg/kg.) used in these experiments (p.26), which corresponds in man to 160mg/70kg man, is a dose which would probably cause a rise of blood pressure and general stimulation of the sympathetic system and this would be accompanied by fright. If this occurred, diminution in appetite would be expected. After one hour when the test of appetite began the animals appeared normal but no observations were made to see if this dose of nicotine did in fact stimulate the sympathetic system of the rat or for how long such an effect lasted. It would, for example, have been easy to see if it caused a rise of blood sugar. It is clear from Fig. 7 that 4mg/kg nicotine completely suppresses food intake but the supposed anorexic activity of 1 & 2mg/kg, in view of the wide scatter, is far from convincing.

Food intake of tolerant rats compared with food intake of fresh rats (Section 10.12.2 (c) sec.)

The statement in the middle of this page that tolerant rats eat less food than fresh rats cannot be accepted unequivocally in view of the fact that litter-mate controls were not used. There is much variation in the amount of food eaten by rats. Moreover tests of significance disregard the effect of the nicotine injections on the emotions of the rats and therefore may be misleading. Dr. Libet in fact showed A.R.A. the results of an experiment carried out to see if tolerant rats ate more food when the daily nicotine injections were stopped. After four days, food intake was increased and so was the food intake of control rats normally injected with saline. This shows that hypodermic injections of even saline result in loss of appetite.

Action of adrenaline and noradrenaline on the appetite test  
(Section IVB, 4(a), p.31)

The difference in the effect of adrenaline and noradrenaline on appetite which appears in the results may be related to the difference in their effect on the blood sugar. Adrenaline under the skin causes a rise of blood sugar which is greatest 2-3 hours after the injection. This rise would inhibit appetite. Noradrenaline is less than  $1/3$  as active as adrenaline on the blood sugar.

With reference to the conclusion following Table 5 (p.30), in which the authors speak of the lasting action of nicotine against food intake, it may be that this action is not only a result of the general stimulation of the sympathetic system, but that it is related to the local effect of the injected nicotine. Dr. Libet, however, did not think that this was possible.

Action of nicotine after the release of noradrenaline  
(Section IVB, 4(b), p.31)

The conclusion drawn in the first paragraph is not justified. Animals after being given reserpine sufficient to remove all catecholamines rarely eat at all and it is possible that the varying figures for the controls in Table 6 (p.31) are explained by variation in the degree of catecholamine depletion. This is borne out by the figures in the last column of Table 6 which are often 0% but sometimes over 100%. 0.5 mg/kg may be an insufficient dose of reserpine in rats; there is no mention of the authority which states that this is a reasonable dose and data should therefore be provided on this point.

Anorectic effect of nicotine on lesioned rats (Section IVB, 5, p.32)

In paragraph 2 of this section the authors describe a weight increase after operation which did not last longer than 2 weeks. If the operation had been successfully carried out the weight increase should have continued. The only conclusion which can be drawn is that the operation was not successfully carried out, although Dr. Libet told L.D. that this particular lesion was easier to perform than lesion of the supratrochlear nucleus.

Action of nicotine on lipid metabolism (Section IVB, 2.33)

The results of this section are shown in Tables 7 & 8. In Table 7 are given figures for epididymal fat; nicotine was without effect after 2 months but caused a decrease from 3 months onwards. It is difficult to judge the significance of the effect because the control figures rose from 373g/100g at 2 months to 447/100g at 3 months. Certainly such results should be determined by the use of litter-mate controls. The figures in Table 8 obviously show a significant difference, but they may be explained by the effect of nicotine in diminishing the amount of food eaten.

The evidence that nicotine releases free fatty acids (FFA) in 10 minutes is likely to be correct because it is known that adrenaline releases FFA in the blood, and nicotine may exert its action by releasing adrenaline.

The evidence that nicotine enhances the disappearance of FFA, the activity apparently being definite after 6 hours (Fig. 10), is interesting. However, Kontinen and Rajasalmi (Unest, *Marin J Clin*, 1953, p. 850) have found that in man the rise of FFA after consumption of fat was the same in 20 smokers as in 20 non-smokers and was even higher at 6 hours than at 4 hours. (The average number of cigarettes smoked by the smokers in 5 hours was 23). The final sentence on p. 37 is therefore not supported by sufficient evidence.

POSSIBLE ACTIONS ON OTHER HYPOTHALAMIC-PIEUTIC FUNCTIONS  
(Section V, p. 36 et seq.)

Investigation of a possible thyrotropic-releasing activity of nicotine on fresh rats (Section VA, p. 38)

Since the authors conclude (top of p. 42) that neither nicotine, adrenaline nor noradrenaline stimulates the hypothalamus to release the factor which leads to an output of thyrotrophic (not thyrotropic) hormone, no comment is required.

Investigation of a possible effect of nicotine on gonadotropic control (Section VB, p. 43)

It may very well be true that large doses of nicotine (p. 44) such as 15mg/kg/day block the action of the gonadotropic (not gonadotrophic) hormone, but clearly such a dose is of no physiological significance.

REPORT NO. 1 REGARDING PROJECT HEMO 11

This report is in effect a literature survey and we do not wish to comment extensively on it. On p. 5, line 4 of the account of the action of tranquillisers the statement is made that reserpine inhibits the electrical activity of hypothalamic structures, particularly those of the posterior nuclei which regulate the sympathetic nerves of the viscera and blood vessels. This statement appears to be in conflict with the evidence of Iggo and Dr. Martin Vogt, 7 R.S. (*Journal of Physiology*, 1960, Vol. 130, p. 144) that there is no distinction in the impulses passing along the preganglionic sympathetic fibres in the animal after treatment with reserpine.

The author has relied a good deal on the views of Brodie (see p. 6) and it should be pointed out that Brodie has changed his views several times in the last few years. Indeed there are so many theories and relatively few facts, that most workers doubt the possibility of any clear description at the present time of the part played by the various substances present in the brain. Thus

Dr. Vogt recently said "The present knowledge about the functional role of the brain constituents (acetylcholine, noradrenaline, serotonin, etc.) is almost nil. This must appear disappointing but the challenge of unsolved problems should stimulate research...." (*Reception Approaches to Psychiatric Problems*, 1962, edited by Tourian, Pollack & Huxley).

#### PROJECT MAPP 11

#### REGULATION OF THE SYSTEMS OF NICOTINE AND OF RESERPINE ON THE HYPOTHALAMIC-PITUITARY AXIS (Section II, 2.3 to 2.4)

It is curious that the mean value for "tolerant controls" and for "tolerant rats" together with standard errors has not been given throughout Table 1. On the face of it, we agree that the values for adrenal weight and for adrenal corticosteroids appear to be greater in the "tolerant rats" but again litter-mate rats should have been used.

#### Action of nicotine on fresh rats subjected to surgical stress (Section III, 7(3), 2.15)

These results show that nicotine in a dose of 1mg/kg, which is large, caused an increase in the release of corticosteroids. This may be due to a release of adrenaline in the first place. There has been no measurement of corticosteroid concentration in the blood (p.19); this is something it is planned to do at Harrogate. We of course do not know whether nicotine, in a dose which could be tolerated, would have a similar effect in man.

#### Action on body weight regulation (Section III, 2.14)

With reference to the action on body weight, which we have already discussed, it would be interesting to compare the effect of nicotine with that of apfetamine, which is a drug that has been used to reduce appetite. Reserpine has not been used for this purpose. (The Battelle workers have in fact done such experiments and in this test apfetamine was active in a similar dose range to nicotine.)

#### REGULATION OF THE SYSTEMS OF NICOTINE AND OF RESERPINE ON BRAIN TRANSMISSION (Section III, 2.24 to 2.25)

We see no reason to accept the conclusion in A, 2 (a) respecting the action of serotonin and the catecholamines in inhibiting the transmission of the nerve impulse in the brain synapse. This is only a theoretical possibility.

#### Effect of reserpine on brain serotonin stores in rats (Section III, 2.12)

It is surprising that the administration of reserpine in so high a dose as 10mg/kg caused only a 50% reduction in the serotonin in the brain of the rat. In other species (e.g. rabbit, Report A37, p.14) depletion approaches 100%. The estimates in Fig. 3 and Fig. 4 are not given. They refer to arbitrary fluorescence intensities.

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Effect of nicotine on brain serotonin stores in rats (Section 1113,  
2(b), 2.501

The observations on the effect of nicotine on the serotonin in the brain are very incomplete, and the conclusion that nicotine "does not act in depleting serotonin stores from brain or other parts of the body" is premature, and contrary to the observations of Wark which the authors themselves quote. It is quite certain that nicotine does not act like reserpine in this respect but it is by no means certain whether there is for example a transient release of serotonin or catecholamines.

FINAL COMMENTS

Of the three main lines of investigation to which these reports have been devoted, we have already commented that the conclusion of the Battelle workers that the anti-diuretic action of nicotine is still present when the supraoptic nucleus is destroyed is almost certainly incorrect. The most interesting results in the reports are on the effect of nicotine on the "stress" mechanism but these by themselves are very incomplete and there is some controversy about them. These experiments should be repeated and supplemented in the T.R.C. Laboratories at Harrogate. Concerning the results on the effect of nicotine on body weight regulation, it would be unwise to conclude that smoking can be used as a means of reducing weight on the evidence presented.

The information of these reports is not sufficiently complete to justify any form of publication.

A.X.A.  
J.H.S.  
21.6.63

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REC'D FROM LND

A. 123

T.I.R.C., NEW YORK

TELEPHONE CONVERSATION WITH MR. MOYT, 26TH JUNE 1961

Battelle Reports on Project "Whisper"

A copy of the three Battelle reports on the effects of nitrogen (Project "Whisper") had been sent by air mail to T.I.R.C. at the request of I.T.C.

Mr. Moyt said that they had not yet had an opportunity to look at the reports on Project Whisper. Even if they should feel it was desirable that the reports should be submitted to the Surgeon General's Advisory Committee, T.I.R.C. would not do that themselves. The reports would have to be presented through Brown and Williamson or some other channel. T.I.R.C. are trying to keep their approach to S.G.A.C. on the highest scientific plane and not coming over backwards to avoid any appearance of presenting propaganda on behalf of manufacturers. Mr. Moyt feels that this approach has worked very well and that T.I.R.C. is held in higher regard by S.G.A.C. than their antagonists who have been in the other direction. Dr. Mackay will go through the Battelle reports but they have no immediate thought of doing anything with them.

Letter to British Committee

Mr. Giese's letter has been sent to the Surgeon General's Advisory Committee.

Timing

It is no longer necessary to put in all evidence to S.G.A.C. by 30th June. All that has been asked is that everything of a broad nature should be put in by that date as two of the members of the Committee are going to spend the summer going through all the evidence in detail. The end of 1961 is now the best estimate for publication of the report.

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Dr. Osborne, who attended a meeting of the American Medical Association on behalf of the Surgeon General, stated that the report might be published either late this year or early in 1964.

Visit by Dr. Little & Mr. Hoyt to U.K.

Mr. Hoyt was proposing to book a sea passage for Dr. Little arriving in the U.K. about 1st September. He himself would fly in about Monday, 3rd September. He stated that we should make no fixed plans as yet for the visit.

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THE FATE OF NICOTINE  
IN THE BODY

Battelle

centre de recherche de Genève

OW-WZ-00000

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III  
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THE FATE OF NICOTINE  
IN THE BODY

for the

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Westminster House  
7 Millbank  
London S. W. 1

by

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May 1963

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Geneva

8W-42-00001

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# in CUMPHREY

## THE FATE OF NICOTINE IN THE BODY

by

H. Geissbühler and C. Haselbach

### INTRODUCTION

There is increasing evidence that nicotine is the key factor in controlling, through the central nervous system, a number of beneficial effects of tobacco smoke, including its action in the presence of stress situations (Larson, Haag & Silvette (1960)). In addition, the alkaloid appears to be intimately connected with the phenomena of tobacco habituation (tolerance) and/or addiction (Larson et al. (1960)). Detailed knowledge of these effects of nicotine in the body of a smoker is therefore of vital importance to the tobacco industry, not only in connection with their present standard products, but also with regard to future potential uses of tobacco alkaloids.

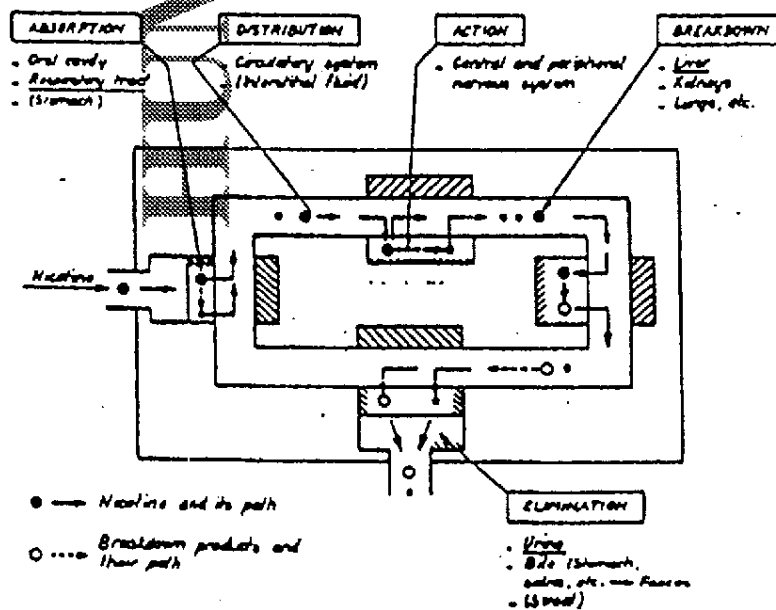
The numerous effects of nicotine in the body may, at first, be conveniently measured by various physiological and pharmacological experiments. However, the elucidation of the mode(s) of action of nicotine will ultimately depend on biochemical analyses dealing with the behaviour of the nicotine molecule on, and its interactions with, the surface of physiologically active, macromolecular cell constituents (enzymes,

receptors, etc.). The success of such analyses depends, in turn, on a detailed knowledge of the fate of nicotine in the body, i.e. of the various mechanisms which control the type and the rate of (a) absorption, (b) distribution, (c) breakdown or transformation, and (d) elimination.

As may be seen from the review of Werle, Schlievelbein and Spieth (1956) and the comprehensive account of literature compiled by Larson et al. (1960), numerous investigators have already examined various aspects of the fate of nicotine in the body. In spite of these efforts, the results are far from being sufficient to allow complete mapping of a certain quantity of nicotine entering the mouth of a smoker. Many of the previous investigations were carried out with chemical methods which are neither sensitive enough to detect the trace quantities of alkaloid involved, nor sufficiently specific to distinguish between nicotine and some of its early breakdown products (for example the cyanogen bromide reaction; Corcoran et al. (1939); Wolff et al. (1948); Tsujimoto et al. (1955)). In addition, when examining certain phenomena as a function of time, the intervals chosen in these experiments very often did not take into account the apparently extremely rapid rates of distribution and breakdown that were to be expected from physiological experiments (Libet & Gerard (1938); Oulitheroff (1952)). Thus both blood and tissue levels of nicotine were measured in terms of hours or even days after administration (Werle & Uschold (1948); Wolff et al. (1949); Gans et al. (1951); Tsujimoto et al. (1955)). Such intervals, while allowing certain deductions as to the rate of elimination of the alkaloid, certainly do not permit any conclusions with regard to blood and tissue concentrations during the period of main physiological activity. It is only very recently that some aspects of the rate of nicotine distribution and breakdown, especially in brain tissues, have been examined in terms of short time intervals (Schmitterlow & Hansson (1962); Hansson & Schmitterlow (1962); Appelgren et al. (1962)).

The present report summarizes and discusses the essential results of an extensive investigation dealing with absorption, distribution, breakdown and elimination of (isotopically labelled) nicotine in human beings and animals. Since the chemical aspects of nicotine metabolism, i. e. the structural modifications brought about by enzymatic transformations, are at present extensively examined by McKennis and his group (Bowman, Turnbull & McKennis (1959); McKennis, Bowman & Turnbull (1960) (1961); McKennis, Turnbull, Schwartz, Tamaki & Bowman (1962); McKennis, Turnbull, Bowman & Schwartz (1962); Bowman & McKennis (1962)), chemical analyses have been limited to the application of methods permitting distinction between the unchanged alkaloid and major labelled breakdown products.

In order to describe the present results in a logical way, the general pathway of nicotine in the body of a smoker is schematically indicated as follows.



According to this scheme, nicotine is carried into the oral cavity and the respiratory tract by smoke particles. A small percentage, which has been suspended or dissolved in saliva, may reach the stomach. After the alkaloid has been absorbed by the tissues lining these tracts, it is transferred into the circulatory system (blood stream), which, in turn, distributes it to the various organs and tissues of the body. There, the alkaloid may provoke its different physiological actions, whereby its contact with the nervous system appears to be of prime importance. In a number of organs, especially in the liver, which is the main detoxifying organ of the body, nicotine is structurally modified (broken down) by enzyme systems. The bulk of breakdown products as well as the small percentage of remaining nicotine are then removed from circulation by the kidneys and eliminated from the body by urine. A small fraction of the compounds may either be secreted from the liver by the bile and thus leave the body by the intestinal tract, or it may be removed from circulation by the salivary and sweat glands.

This sequence of the various phenomena will be more or less followed in describing the results of the present investigation.

## RESULTS

### Amounts of nicotine absorbed in cigarette smoking

The absolute quantity of nicotine absorbed upon smoking of a single cigarette depends (a) on the nicotine present in the tobacco, (b) on the amount of alkaloid transferred into the main stream smoke (or the smoke drawn into the mouth by the smoker), and (c) on the percentage of main stream nicotine absorbed by the smoker. These factors have been examined on a representative number of confirmed smokers by applying more refined and standardised techniques than

were used by previous investigators (for technical details, see Appendix).

The group of human subjects investigated consisted of six males and four females of varying smoking habits and histories (see Table 1).

In a series of experiments, factor (c), i.e. the percentage of absorption, was determined. The ten subjects smoked identical weight and draw selected cigarettes which had been fortified with (methyl)-C<sup>14</sup> nicotine hydrogen tartrate\*. This procedure allowed analysis by chemical means and double-checking by radio-isotope determination. The results are presented in Table 1. Good agreement between chemical and isotope measurements was observed. The data demonstrate that the percentage of absorption varied within wide limits, ranging from 20 % to 80 % in terms of nicotine drawn into the mouth. The two non-inhalers (W.S. and H.G.) absorbed 42 % and 22 % respectively. Whereas the majority of smokers who inhale absorbed more than 70 %, two subjects (P.R. and J.P.) remained considerably below this level (40 % - 30 %). Both of them eject the smoke immediately after inhalation.

These results demonstrate that there is no distinct limit separating inhalers from non-inhalers with regard to the percentage of nicotine absorbed. Although the whole group of inhalers averaged 75 % absorption, two individuals retained no more alkaloid than one of the non-inhalers. It remains to be determined whether a given percentage of nicotine absorbed by a non-inhaler is physiologically as active as the same percentage absorbed by an inhaler (see elimination experiments for further information).

In a second series of experiments, the same technique was applied to measure the average quantity of nicotine which is transferred from the burning cigarette into the main stream smoke by each individual smoker (factor b). In order to determine the actual amounts of nicotine which they usually draw into their mouth, the subjects this time smoked their regu-

\* All radio-active nicotine used in the present investigation was labelled in the methyl-position.

lar brand cigarettes. Smoking was carried out according to individual habits with regard to puff-rate and -volume, number of puffs per cigarette, and length of cigarette smoked. These measurements were completed by analyzing the different brands of cigarettes for their nicotine content <sup>\*)</sup> (factor a). From these data and the absorption percentages presented above, the average quantity of nicotine absorbed by each smoker upon smoking of one cigarette was determined. The results are presented graphically in Fig. 1. They demonstrate that the quantities of nicotine drawn into the mouth by individual smokers were strikingly different, ranging from about 1 mg to more than 5 mg per cigarette. These amounts of nicotine transferred into the main stream smoke do not parallel the nicotine content of the various cigarettes, but apparently are due to markedly differing smoking habits of the subjects. In fact, except for the two non-inhalers, individual amounts of nicotine absorbed are much more affected by the quantity of alkaloid transferred than by the percentage of absorption. The absolute quantities absorbed per cigarette vary between 0.5 and 3.5 mg. It is interesting to note that both non-inhalers compensate the low percentage of absorption by a relatively high rate of transfer.

The average daily quantities of nicotine absorbed by each smoker, as calculated from the data in Fig. 1, are presented in the last column of Table 1. Four of the ten smokers (2 males, 2 females) absorb amounts of nicotine in excess of 80 mg each day, four of them consume approx. 40 mg, and two of them get smaller quantities.

Three of the subjects investigated (C. H., P. F. and H. G.) had already participated in earlier experiments, in which daily nicotine consumption had been measured by a completely different approach. In these experiments, the average daily quantity of nicotine eliminated

<sup>\*)</sup> Analyses carried out by the Research and Development Establishment, The British American Tobacco Co. Ltd., Southampton, England.



In the urine was multiplied by a factor of ten assuming that approx. 10 % of the alkaloid absorbed had been eliminated chemically unchanged (Stekennis (1960)). These earlier results are also included in Table 1. They agree quite well with the data obtained by direct absorption-analysis.

The group of subjects participating in the elimination-type experiments consisted of 21 individuals (all males). Their average amount of nicotine absorbed per day, as calculated from the quantity of unchanged alkaloid eliminated in the urine, are shown in Fig. 2. The graph distinguishes between inhalers and non-inhalers, and between those smoking more and those smoking less than 30 cigarettes per day. For the group as a whole, the daily nicotine consumption is of the same order of magnitude as that of the group which was subjected to direct absorption measurements. Again, the individual amounts of alkaloid absorbed vary within wide limits, ranging from less than 1 mg to 70 mg/day. However, there is a distinct difference between heavy smokers (30 and more cigarettes/day), who, with one exception, absorbed more than 20 mg/day, and medium to light smokers (less than 30 cigarettes/day), the majority of which consumed less than 20 mg/day. Furthermore, none of the non-inhalers absorbed more than 35 mg of alkaloid per day.

Of the 21 male subjects investigated during the elimination-type experiments, 11 smoked non-tipped, and 10 adhered to filter-tipped cigarettes. Comparison of the two groups shows that the former absorbed on the average 34.3 mg, the latter 17.3 mg of nicotine per day. Statistical evaluation showed this difference to be significant (probability of error  $\alpha_{1-t} = < 25\%$ ). Although nicotine transfer could not be measured in these experiments, observations indicate that diminished absorption upon regular smoking of filter-cigarettes is due not to the efficiency of filters in removing nicotine but rather to differing smoking habits (especially as regards the average length of cigarette smoked).

# produced by RJRTC

Table I

Smoking habits of ten individual smokers and their rates of nicotine absorption as determined under standardized smoking conditions (for details, see Appendix)

Subject	Sex	Age	History/habits			Nicotine absorption %		Daily nicotine consumption mg/nicotine/day	
			Y	D	T	c	r	a	b
C. H.	m	39	24	45	i.	87	85	81	48
P. F.	m	40	23	27	i.	80	86	81	40
J. P.	m	43	23	21	i.	95	94	47	-
P. R.	m	42	22	46	i.	48	48	22	-
W. S.	m	42	26	20	p.	41	42	35	-
H. Q.	m	37	16	25	n.	21	23	33	35
L. G.	f	40	35	20	i.	89	89	73	-
B. N.	f	36	16	18	i.	77	70	21	-
J. P.	f	39	17	15	i.	36	42	10	-
H. F.	f	36	13	31	i.	95	96	62	-

## Legend:

History/habits: Y = years of continuous smoking; D = daily consumption of cigarettes;  
T = type of smoking (i = inhales; n = does not inhale, according to smoker's opinion).

Nicotine absorption: expressed in % of nicotine in main stream smoke (c = chemical; r = radio-chemical analysis).

Daily nicotine consumption: a) Calculated from absorption data obtained by smoking regular brand cigarettes under standard conditions.

b) Calculated from analysis of daily quantity of nicotine eliminated in urine (multiplied by a factor of 10).

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Distribution of nicotine within the body by the  
circulatory system

The primary physiological effects of the particular quantities of nicotine that are absorbed upon smoking, the magnitude of which has been discussed in the foregoing section, depend on the rates with which the alkaloid is transferred from the sites of absorption into the blood stream and subsequently distributed to the various sites of action in the body.

In the present experiments, the rate of distribution was determined by measuring radio-activity in blood and different tissues as a function of time after intravenous injection of  $C^{14}$ -nicotine diphosphate (results to be discussed in the present section).

The approximate rates of transfer from the absorbing site(s) were then determined by comparing distribution data after intravenous administration with those obtained upon pulmonary (and gastric) absorption of the labelled alkaloid (results presented in subsequent section). Since the detection of trace amounts of radio-activity in blood and tissues of living human beings was technically not feasible, distribution- and transfer-experiments were carried out on laboratory animals.

It is evident that the enzymatic breakdown of nicotine in the body starts immediately after absorption (Werle (1938); McKennis (1960); Hansson & Schmitzerlow (1963)). In order, therefore, to measure the actual concentration of the unchanged alkaloid, the total radio-activity recovered from blood and tissues had to be fractionated into nicotine and breakdown products.

A first series of experiments was carried out on anaesthetized rabbits which were injected with small quantities (0.1 - 0.2 mg base/kg) of  $C^{14}$ -nicotine. Blood levels of total radio-activity and unchanged nico-

time, as observed in a typical short-time experiment, are shown in Fig. 3 (a). The curves demonstrate that the alkaloid was very rapidly removed from the blood stream, more than 90 % of the radio-activity injected having disappeared in less than 5 minutes. Then the curves leveled off, and further removal proceeded at a much lower rate. After a longer observation period, approximately 2 % of radio-activity injected were still present in the blood (Fig. 3 (b)). Fig. 3 further demonstrates that nicotine was broken down in the rabbit body at a high rate. Ten minutes after injection, only about 30 % of the total radio-activity circulating in the blood represented unchanged alkaloid. After 4 hours, no more nicotine was detectable.

Total radio-activity in various organs (expressed on the basis of tissue water) after various time intervals is shown in Fig. 4. The results demonstrate that 5 minutes after injection, the concentration of radio-activity was much higher in brain, liver, lungs, and especially in kidneys, than in blood. At later time intervals, the differences had diminished; however, there was still more radio-activity in the  $C^{14}$ -eliminating organs (liver, kidneys, lungs) than in the circulatory fluid. In heart and muscle tissue, total radio-activity was found to be about equal to that in blood at all time intervals examined. Label recovered from fat deposits (adipose tissue) was extremely small, amounting to no more than 10 % (on a weight basis) of the concentrations measured in blood.

Total radio-activity recovered from various organs was further fractionated by homogenizing the tissues, precipitating proteins and extracting the supernatant with chloroform (for details, see Appendix). Radio-chromatograms of the concentrated chloroform extracts regularly revealed two compounds (nicotine and cotinine), and sporadically a third one (most likely hydroxycotinine). Typical radio-chromatograms are presented in Fig. 9. These chromatograms were evaluated quantitatively in order to determine the relative quantities of the substances detected.

The results of a typical experiment showing the relative concentration of nicotine and the chloroform-soluble breakdown product(s) in various tissues as a function of time are shown in Fig. 5<sup>\*)</sup>. Thirty minutes after injection, nicotine was more abundant than cotinine in all tissues analyzed. After 80 minutes, the concentrations of the two compounds were about equal, except in kidneys and lungs, which still contained more nicotine. Two hours after administration, cotinine was more abundant than nicotine. After eight hours, the two organs which still had sufficient radio-activity to be fractionated (liver, kidneys) showed no more nicotine. On the other hand, both contained measurable quantities of hydroxycotinine. It ought to be mentioned that the chloroform-soluble fraction derived from tissues comprised only 40 % - 80 % of their total radio-activity, thereby indicating the presence of breakdown products not extracted by this solvent. The percentage of the chloroform-insoluble fraction generally increased with increasing time intervals. However, even 30 minutes after injection, nicotine detected on radio-chromatograms represented no more than 50 % of the total radio-activity except in kidneys, in which it comprised 85 %.

So far, the results of blood and tissue analyses demonstrate that upon its introduction into the circulatory system, nicotine is immediately partitioned between blood and tissues. This period of partitioning, which is represented by the first, steep part of the concentration-vs-time curve (Fig. 3), is terminated within a few minutes in rabbits. In the tissues, nicotine is enzymatically modified at various rates. The further concentration of the alkaloid and its breakdown products in blood appears to be governed by the rates at which they are released back into the circulatory system and/or the rate with which they are removed from blood by the various eliminatory mechanisms. This period, which is represented by the second, flat part of the concentration-vs-time curve, lasts more than 8 hours in rabbits. As regards the rate of enzymatic breakdown of the alkaloid, the tissue analyses indicate that in rabbits, its half-life is no more, and in some organs even less, than 30 minutes.

\*) With the heptane-extraction procedure used for blood, breakdown products (especially cotinine) could not be measured. Their absence in Figs 5 and 8 is therefore without significance.

The experiments discussed so far have been concerned with small quantities of nicotine administered. It remained to be determined whether or not the picture of distribution and breakdown is drastically changed when injecting higher quantities of alkaloid (differing by a factor of at least 1 : 10). This second series of experiments was carried out by using the isotope dilution procedure. Fig. 3 (c) demonstrates that no significant change in the pattern of partitioning occurred upon injection of a higher quantity of nicotine. On the other hand, significant differences in the concentration of unchanged alkaloid were observed for the second (flat) part of the curve. From these data, it appears that the mechanism regulating the flat portion of the blood concentration curve (breakdown and/or elimination) became saturated.

This assumption was supported by tissue analyses (Fig. 6). The results show significant differences in the composition of the chloroform-soluble fraction 80 minutes after administration. For example in the liver of the animal having received the lowest dose, the concentration of cotinine was much higher than that of nicotine. The opposite was true for the animal injected with the highest dose, animal whose liver contained much more nicotine than cotinine. Essentially the same pattern was found in the kidneys and brains, whereas the lungs showed little difference.

The results clearly demonstrate that with the higher dose of nicotine injected, accumulation of the alkaloid occurred in three of the four organs investigated. This phenomenon may only be explained by saturation of the breakdown and/or excretory mechanisms.

The influence of the absorbing site on the  
pattern of nicotine distribution

The foregoing experiments have been concerned with the distribution of nicotine and its accumulation in tissues once it had been introduced into the blood stream. When its administration is carried out in any other way (subcutaneous, intramuscular, gastro-intestinal, etc.), the concentration of the alkaloid in blood as a function of time, and therefore its pattern of distribution, becomes dependent on the rates with which the alkaloid is absorbed by the particular tissues and subsequently released into the blood stream.

In view of the practical importance of present and possible future types of nicotine consumption by human beings, the present investigation has been concerned with a preliminary study of nicotine uptake by the stomach and with more extended experiments dealing with the rate of absorption of the alkaloid in the respiratory tract upon smoking.

Pharmacological and physiological experiments have already shown that the absorption of nicotine by the stomach mucous membranes and its subsequent release into the blood stream proceed at a slow rate owing to the highly acid conditions prevailing in the stomach cavity (Travall (1940) (1960)). Upon increase of the gastric pH, nicotine apparently permeated more readily since its effects were more pronounced and accelerated.

To our knowledge there has been no systematic investigation of the rate of transfer of nicotine after absorption of tobacco smoke in the respiratory tract. There are some preliminary observations indicating that the free alkaloid, when administered in a vapour or mist, penetrates lung tissue much more rapidly than do its salts (Larson (1960)).

In the present experiments, distribution after gastric absorption was followed after introduction of  $C^{14}$ -nicotine hydrogen tartrate into the buffered stomach lumen of rabbits. For investigating pulmonary absorption, the rabbits were exposed to tobacco smoke fortified with the labelled alkaloid. This smoke, which was generated by having anaesthetised animals puff cigarettes by their own respiratory activity, was introduced directly into the trachea (for details of apparatus, see Appendix). The concentration of radio-activity in blood and tissues was followed in the same way as in previous experiments.

Fig. 7 compares the blood levels of total radio-activity and unchanged alkaloid after gastric and pulmonary administration with those observed after intravenous injection. The data demonstrate that transfer of nicotine into the blood from the stomach proceeded at a low rate even under buffered conditions. In addition, the percentage of radio-activity representing unchanged alkaloid dropped to a low level after a relatively short time interval.

On the other hand, nicotine was rapidly transferred into the blood stream after absorption from tobacco smoke. Its concentration in blood in terms of the total quantity absorbed came close to that observed a few minutes after intravenous injection, i. e. at the point where partitioning of the injected quantity was terminated. It would appear that upon smoking, nicotine is transferred into the blood stream at a rate which is close to that with which the alkaloid is partitioned.

As regards the concentration of radio-activity accumulating in various tissues, it was observed that upon gastric absorption, radio-activity was very low in all organs examined except in the liver. Of particular interest is the fact that brain concentrations stayed very low during the whole duration of the experiment, reaching no more than about 5 % of the level observed after intravenous injection. In addition to the difficulties in getting nicotine across the stomach mucous membranes, it appears that the particular location of the stomach favours the direct transport of the alkaloid to the liver, where it is metabolised at a high rate.



Tissue levels of nicotine and chloroform-soluble breakdown products shortly (2 - 3 minutes) after smoking was stopped are shown in Fig. 6. The results demonstrate that concentrations of radio-activity in terms of the quantity absorbed were comparable to those observed after intravenous injection. Of particular interest is the high accumulation of label in the brain. The data further show that breakdown of the alkaloid had already started within the short period of investigation. Radio-activity representing metabolites was especially high in the lungs, thereby indicating that these substances had not been carried to respiratory tissues by circulation, but that the organ had actively participated in their formation. There is a difference between rabbits A and B as regards the relative amounts of unchanged nicotine and metabolites recovered from their lungs, indicating that individual variations in the rate of breakdown may occur. Total radio-activity present in the lungs at the end of the smoking period was of the order of 4 % of the quantity absorbed, which fact further demonstrates the rapid disappearance of nicotine from this tissue.

Blood and tissue analyses carried out during and after smoking show that in contrast to absorption through the stomach, nicotine present in smoke particles is rapidly transferred through the pulmonary membranes. Once it has entered the blood stream, it is distributed and metabolized according to the pattern established in the foregoing section.

### Elimination of nicotine and its metabolites

Investigations which have dealt with the excretion of nicotine and its breakdown products are numerous (Cans et al. (1951); Bennet et al. (1954); Owen & Larson (1958); Truhaut & de Clercq (1959); McKennis (1960); McKennis et al. (1961); Hansson & Schmitterlow (1962)). These investigations have been carried out on human beings (smokers and non-smokers) and on a variety of different animals, including dogs, cats, rabbits, rats and mice. There is general agreement that the substances are eliminated mainly through the urine and that about 10 % of the total or urinary elimination products represent unchanged nicotine. This percentage of unchanged nicotine appears to vary with the pH of the urine and also with the dose of alkaloid absorbed (Hans & Larson (1942); Finnegan et al. (1947)).

The chemical structures of the metabolites which so far have been isolated from human and dog urine by McKennis et al. indicate that the general pathway of nicotine breakdown is quite similar in these two species (Bowman & McKennis (1962)). The metabolic routes which apply to humans and dogs appear to be applicable in part to other animals (Truhaut et al. (1959); McKennis et al. (1962); Hansson & Schmitterlow (1962)). In the report of Truhaut & de Clercq (1959) dealing with nicotine metabolism on rats, breakdown products (especially dihydro-metanic) have been described which suggest the possibility of important intramammalian species differences. However, these results have not so far been substantiated by other authors.

A small part (2 - 3 %) of eliminated products leaves the body with the faeces, and it appears that this part is composed of substances secreted by the bile, by salivary glands and by the stomach or intestinal membranes (Hansson & Schmitterlow (1962)). Besides there are some indications of rather indefinite nature that a small part of nicotine is excreted by the sweat glands (Larson et al. (1960)).

Most recent elimination experiments have been carried out with isotopically labelled nicotine, and it is evident that the percentages of radio-activity, in terms of the quantity administered, which are excreted in the urine, in faeces or by any other eliminatory mechanism, depend on the particular position of the labelled atom(s) on the alkaloid molecule. For example, more than 90 % of the activity of randomly labelled alkaloid was found to be eliminated in the urine (+ 2 % in faeces) of various animals, whereas with the compound labelled in the methyl group, urinary elimination was of the order of 60 % - 70 % (+ 4 - 6 % in the faeces). Moreover, between 6 and 10 % of radio-activity derived from the latter compound left the animal body as radio-active  $\text{CO}_2$  with the expired air (Ganz et al. (1951); Bennet et al. (1954); McKennis et al. (1961) (1962); Hansson & Schmiterlow (1962)).

In the present experiments, elimination of radio-activity in rats, rabbits and smokers was compared in order to obtain some indications on species differences which might exist with regard to the rate of elimination, the pathway of elimination and the rate of breakdown of nicotine. In addition, elimination of nicotine and breakdown products by rats having a different physiological disposition towards the alkaloid was investigated. Finally, urinary removal of radio-activity by smokers was examined with regard to possible relationships between the average amount of nicotine absorbed and the rate of elimination.

Total radio-activity eliminated in urine during a 30-hour period averaged  $85 \pm 3$  % for rabbits and  $87 \pm 5$  % for rats. In human beings, which had absorbed the labelled alkaloid by smoking cigarettes fortified with  $\text{C}^{14}$ -nicotine hydrogen tartrate, the radio-activity eliminated in urine within 30 hours varied within wide limits, ranging from less than 10 % to more than 90 % in terms of the quantity absorbed (Fig. 11). Fig. 10 demonstrates that there is no significant difference between the two animals and between these and smokers with regard to the rate of elimination (total radio-activity eliminated per hour), peak

elimination occurring within the first hour after administration. Elimination of total radio-activity was somewhat delayed in rabbits (maximum between 1 and 4 hours). However, it appears that this delay was mainly due to the antidiuretic effect of the injected nicotine (1.0 mg/kg), which almost stopped urinary flow during the first hour. In fact, it was found with several groups of rats, which had received 1-2 mg/kg of alkaloid and whose urinary flow had not been secured by prior administration of large amounts of water, that the rate of radio-activity eliminated became dependent on the rate of urination. With smokers, no such correlation between radio-activity eliminated and rate of urination was observed.

Further fractionation by radio-chromatography of total radio-activity eliminated in urine revealed a number of labelled compounds, some of which corresponded to those described by McKenna et al. Typical chromatograms of fractionated rat, rabbit and smoker's urines are shown in Fig. 9.

Urine collected from rats at various time intervals after injection regularly contained three labelled compounds whose solubility and chromatographic behaviour in various solvent systems corresponded to that of authentic samples of nicotine, cotinine and hydroxycotinine<sup>1)</sup>. In addition, chromatography of unprocessed rat urine normally revealed several unidentified peaks, one of which was particularly prominent in early (3- and 8-hour) samples (see Fig. 9). This peak could not be extracted into chloroform under either acid or alkaline conditions. Furthermore, its *R<sub>f</sub>*-values did not correspond to those of authentic  $\gamma$ -(3-pyridyl)- $\gamma$ -methyl-aminobutyric acid, which compound was found to be an early breakdown product in dog urine (Bowman et al. (1959)). Rabbit urine contained nicotine, cotinine, hydroxycotinine and at least five unknown metabolites which were not further examined. Total radio-activity in urine of smokers was too low to be analysed without prior concentration. Samples were therefore extracted

<sup>1)</sup> The authors are very grateful to Dr H. McKenna Jr., Department of Pharmacology, Medical College of Virginia, Richmond, Va., USA, for his supplying samples of a number of nicotine metabolites.

with chloroform under alkaline conditions, and the concentrated  $\text{CHCl}_3$ -extract subjected to chromatography. This fraction, which comprised between 95 % and 15 % of the total radio-activity, again showed nicotine, cotinine and hydroxycotinine. Sporadically one to two minor unidentified peaks were observed. Based on these qualitative chromatographic results, total radio-activity of the various urine samples collected from rabbits, rats and smokers was divided into radio-activity representing nicotine, cotinine, hydroxycotinine and unknown, whereby the unknown fraction from smoker's urine was represented by the chloroform-insoluble labelled material (Figs 12 and 14).

Fig. 12 compares the three species with regard to the average composition of total radio-activity eliminated in urine as a function of time. The relative amounts of nicotine and metabolites eliminated at the various time intervals, especially shortly after administration (3-hour samples), indicate that breakdown of the alkaloid was slightly more rapid in rabbits than in rats and that the metabolism was slower in smokers than in either of the two animals. In addition, the data show that the cotinine-hydroxycotinine pathway was more prominent in rabbits than in rats or smokers.

A further species difference between rats and rabbits was observed with regard to the percentage of radio-activity eliminated as respiratory  $\text{C}^{14}\text{O}_2$  (Fig. 13). The shape of the elimination curve (radio-activity eliminated per hour plotted against time) was found to be similar for the two animals, peak concentrations occurring within the first three hours after administration. However, in terms of radio-activity injected, the percentage of label eliminated in the expired air was considerably higher in rabbits than in rats. It would therefore appear that either the demethylation of cotinine or the breakdown of methylamine, both of which presumably are involved in the formation of labelled  $\text{CO}_2$  from (methyl)- $\text{C}^{14}$ -nicotine (McKennis et al.

(1961) (1962)), is a more important pathway in rabbits than in rats. In this connection it is interesting to note that Bowman & McKenna (1962) were unable to find demethylcotinine in human urine after administration of nicotine or cotinine. These results indicate that demethylation might be much less important in smokers than in either rabbits or rats.

Further elimination experiments were carried out on rats having a different physiological disposition towards nicotine. Tolerant rats, i.e., animals showing no physiological response to a given nicotine concentration in the pivoting test, were obtained by prolonged exposure to the alkaloid. There was no difference between tolerant and control rats with regard to the rate of urinary elimination of total radio-activity. However, there was a difference between the two types of animals in the relative amounts of nicotine and metabolites which they eliminated shortly after injection (Fig. 14). In 3- and 8-hour urines of tolerant rats, the relative amount of nicotine was considerably smaller than in non-treated ones, thereby indicating that breakdown of the alkaloid was more rapid in the former animals. In the same experiments it was observed that rats (controls as well as tolerant animals) showed a distinct sex difference with regard to the relative amounts of nicotine and metabolites eliminated, thus suggesting that males detoxify nicotine more rapidly than females (Fig. 14).

A number of preliminary experiments were conducted in order to determine whether similar differences in the rate of breakdown of nicotine existed in different types of rabbits. These degradation experiments were carried out with a liver microsomal system which was prepared according to the method of Hucker et al. (1960) and Gillette (1959). Liver preparations derived from non-treated male and female rabbits, and from animals exposed to alkaloid for prolonged periods were fortified with reduced nicotinamide adenine dinucleotide phosphate (NADP) and glucose-6-phosphate, and were

incubated under aerobic conditions with (methyl)- $C^{14}$ -nicotine for periods of up to 4 hours. Rates of nicotine breakdown, as measured by the disappearance of the unchanged alkaloid and by radio-chromatography, were found to be similar to those observed by Hucker et al. However, there was no significant difference in activity between liver preparations derived from the various types of rabbits. Thus the results obtained in elimination experiments with rats could not be substantiated with the more specific detoxifying system derived from rabbits.

The enormous variation in the percentage and rate of urinary elimination of radio-activity by smokers after inhaling tobacco smoke (profiled with (methyl)- $C^{14}$ -nicotine has been briefly noted before. A small number of smokers selected from the whole group was therefore subjected to more than one experiment (Fig. 11; subjects C.R., H.F., L.O. and W.S.). Fig. 11 demonstrates that individual rates and percentages of elimination were quite consistent. Consequently, it does not appear that these elimination data were significantly affected by external conditions (pH and volume of urine, etc.). In addition, it was found that the rate of elimination of radio-activity did not depend on the amount of additional nicotine consumed during the observation period. (In their second experiment, subjects H.F. and W.S. did not smoke after absorption of  $C^{14}$ -nicotine.)

Six of the ten smokers who participated in these experiments eliminated amounts of radio-activity sufficient to be fractionated. Radio-chromatography of the chloroform-soluble fraction of individual urine samples demonstrated that the subjects varied considerably with regard to the relative quantities of unchanged nicotine and metabolites eliminated. However, in terms of the quantity of nicotine absorbed, the individual amounts of unchanged nicotine eliminated came surprisingly close to the expected value of 10 % (individual values = 9.2, 15.8, 11.0, 7.8, 6.0 and 9.5; average = 9.9 %). The limited results of elimination analyses available do not point to any difference in the rate

of breakdown of nicotine between male and female smokers, nor do they establish any relationship between the rate of breakdown and the average daily quantity of nicotine absorbed. The present results point to the existence of a relationship between the daily quantity of alkaloid absorbed and the rate at which total radio-activity is eliminated. However, the number of persons participating in the experiment was too limited to make it statistically significant.

Of particular interest is the fact that both non-inhalers (W.S. and H.G.) eliminated low percentages of radio-activity in the urine during the 30-hour observation period. Smoker W.S. was subjected to a prolonged experiment, and was found to eliminate measurable quantities of radio-activity for a period of 120 hours. From this observation it would appear that in non-inhalers, the absorption and distribution of nicotine, and consequently its elimination, proceed at a much slower rate than in inhalers. These preliminary data suggest that non-inhalers, although absorbing similar quantities of nicotine as certain types of inhalers, are exposed to much smaller initial concentrations of the alkaloid owing to initially different pathways and rates of absorption and distribution. The few data available on the rate of absorption of nicotine through the mucous membranes of the oral and nasal cavities indicate that it proceeds at a much slower rate than through the alveolar system of the lungs (Travell (1940)).

In a final series of experiments, the total balance of radio-activity was established with rats and rabbits 30 hours after injection of  $C^{14}$ -nicotine. The results are presented in Fig. 15. They demonstrate that, in addition to radio-activity eliminated in the urine and in the expired air, significant amounts were excreted with the faeces. Fractionation of faeces showed that in early samples (0 - 10 hours after injection), cotinine was the principal metabolite excreted. Unchanged nicotine was considerably less abundant. In later samples (10 - 30 hours), the major portion (85 % - 95 %) of total radio-activity



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could no longer be extracted with chloroform under either alkaline or acid conditions. The same was true for radio-activity recovered from the stomach and intestinal contents upon termination of the experiment. Roughly 10% of the quantity of radio-activity administered was still in the body at the end of the 30-hour observation period. This was to be expected in experiments in which the methyl-labelled alkaloid was used. It would appear that the labelled methyl-group, once removed from the pyrrolidine ring by demethylation, ought to be channelled into the general metabolism of the body either by transmethylation reactions or as  $\text{CO}_2$  after oxidation. In any case, no measurable amounts of chloroform-soluble radio-activity were recovered from tissues 30 hours after administration.

### DISCUSSION

#### Distribution of nicotine in the body

After intravenous administration, nicotine disappeared from rabbit blood in two phases. The first phase was extremely rapid with a half-life of less than one minute. The second phase proceeded at a much slower rate with a half-life of the order of 3 to 4 hours. It appears that the primary factor responsible for this pattern is the rapid binding of nicotine to tissue constituents, and this binding is maintained for a period of hours. The first phase of the disappearance of nicotine from blood is thus due to accumulation in tissues, while the second one is controlled by its slow release, its enzymatic breakdown and, to some extent, its direct excretion.

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The assumption that nicotine is bound to certain cell constituents is supported by the fact that shortly after administration, its concentration in most organs investigated is much higher than in blood. Further evidence is provided by the results of Hansson & Schmiterlow (1962) who showed with histo-autoradiograms that, five minutes after injection,  $C^{14}$ -nicotine had accumulated in the walls of blood vessels. This behaviour of nicotine was in many ways predictable on the basis of its physico-chemical properties. Its lipophilic nature permits rapid absorption, and its penetration into cell membranes (Bacq et al. (1961)). Furthermore, nicotine has a relatively favourable pKa-value at the pH-conditions prevailing in the blood (about 40 % being present in the neutral, non-dissociated form, which penetrates into cell membranes much more easily than electrically charged ions). In addition, nicotine lacks the particular functional groups (such as hydroxyl- or carboxyl-groups) which are involved in the formation of more voluminous, less penetrating molecules by such reactions as hydration, or conjugation with acids.

The high lipid solubility of nicotine is most likely of importance with regard to its physiological and pharmacological activities, since it permits passage of the blood-brain barrier and penetration of the alkaloid into brain and other nervous tissues. Appelgren et al. (1962) and Hansson & Schmiterlow (1962) observed accumulation of radioactivity in the brain and adrenal medulla of rats, and in the central nervous system of cats within a few minutes after administration of  $C^{14}$ -nicotine.

It would appear that partitioning of nicotine between blood and particular tissues is controlled not only by the physico-chemical properties of the alkaloid, but also by the differential rates of blood flow into various organs. According to Wright (1958), the two human kidneys receive 1300 ml of blood/min. of the total cardiac resting output of 5000 ml/min., whereas the blood flow to the brain is estimated

at about 750 ml/min. Blood flow to resting muscles, which represent 40 % of the body weight, is much slower, amounting to no more than 1000 ml/min. or 4 ml/min./100 g of muscle. It is evident that such differences in the rate of blood flow ought to contribute to the differential distribution of radio-activity to various organs as observed immediately after administration of the labelled alkaloid (see Fig. 5).

#### Breakdown of nicotine in the body

The localization of nicotine in many organs does not prevent the immediate degradation of the alkaloid. The rapid appearance of metabolites in most tissues strongly suggests that they were (at least in part) formed in these tissues, and not distributed to them by the circulatory system.

Breakdown of nicotine in isolated tissues other than liver has been observed by several authors (Werle & Müller (1941); Werle & Uschold (1948); Müller & Larson (1953)). During the present investigation, a number of preliminary experiments were carried out with microsomal preparations derived from rabbit lungs and kidneys. Although rates of breakdown were relatively low compared with liver preparations, formation of measurable quantities of cotinine was consistently observed.

Therefore, the enzymatic transformation "in situ" appears to be the main factor in controlling the duration of the physiological activity of the alkaloid in some organs. Of particular interest in this connection is the rate of breakdown in the brain. Hansson & Schmitzlow (1962) followed the appearance of cotinine in the brain of mice and rats, and found similar or even faster breakdown of nicotine than was observed in the present experiments on rabbits. This is in agreement with the results of Larson et al. (1949), who determined the rate of

"elimination" (elimination meaning breakdown and removal) by physiological methods in the dog, cat, mouse and rabbit. They found that rabbits "eliminated" nicotine 1.7 times as fast as the dog, whereas the relative rates for cats and mice (compared to dogs) were 3 and 2.3 respectively. Since the rate of breakdown of nicotine appears to be the decisive factor in controlling the duration of its physiological action, it would be of great importance to know the speed with which enzymatic transformations proceed in the human being. From the present analysis on radio-activity eliminated in human urines, it seems that the average smoker breaks down nicotine at a rate about half of that demonstrated with rabbits, i. e. the average half-life of nicotine in the body of a smoker would be of the order of 30 minutes to 1 hour (based on relative amounts of unchanged nicotine and breakdown products as demonstrated in Fig. 12).

In view of the fact that certain polycyclic hydrocarbons accelerate the metabolic breakdown of a number of drugs in the liver by increasing the activity of microsomal enzymes (Conney & Burns (1962)), it would be interesting to verify if the continued absorption of hydrocarbons upon regular smoking has a similar effect on the rate of breakdown of nicotine.

#### Tolerance and addiction

According to Eddy (1955), the phenomenon of drug tolerance may be explained by one of the following mechanisms: (a) decreased absorption, (b) change in the rate of excretion, (c) altered tissue distribution, (d) altered rate of metabolic transformation, and (e) cellular adaptation.

Taking as a basis the results obtained with rats, we were first inclined to believe that tolerance to nicotine might be connected with an accelerated rate of metabolism. Similar explanations of tolerance to certain drugs, including barbiturates, meprobromate, etc., have

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been advanced by a number of authors (Remmer (1959); Cooney et al. (1960); Phillips et al. (1962); Cooney & Burns (1962)). However, most of these experiments have been limited to rats and have not yet been extended to other animal species. Rats might well be as unusual animals with regard to changing rates of biotransformations as they are with regard to sex-differences in the rate of breakdown of drugs (Cooney & Burns (1962)). In addition, a careful quantitative evaluation of the rates of breakdown, as observed in urinary elimination experiments, indicate that the differences between non-treated and tolerant rats are far from being sufficient to account for the striking differences in the physiological behaviour of the animals.

Although tolerance to some drugs may depend on accelerated enzymatic breakdown, prolonged consumption of others, including morphine, appears to induce cellular adaptations (Axelrod (1956); Shuster (1961); Takemori (1961) (1962)). In any case, the present results offer no conclusive evidence for any particular mechanism involved in tolerance to nicotine, nor do they indicate a lead to the phenomenon of addiction. We believe that both tolerance and addiction are intimately connected, and that it would be most useful to investigate the two phenomena with regard to cellular adaptation, especially in target organs of the central nervous system.

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## APPENDIX

(Experimental Methods)

### Smoking experiments

Regular weight- and draw-selected cigarettes (British American Tobacco Co. Code No. 15, 70 x 35 mm,  $1.02 \pm 0.02$  g, pressure drop =  $1.0 \text{ mm H}_2\text{O} \pm 4\%$ , 12.5 % moisture, 1.53 % nicotine) were fortified with (methyl)- $\text{C}^{14}$ -nicotine hydrogen tartrate (0.15 - 0.5 mg base). For injection of the labelled alkaloid solution, cigarettes were mounted on a special cigarette injector apparatus so arranged as to deliver 40  $\mu\text{l}$  of solution uniformly along the whole length of the cigarette<sup>1)</sup>. The amount of radio-activity actually injected was verified by steam distillation of treated tobacco of several cigarettes of each series and subsequent measurement of the distillates as outlined below.

Smoking of labelled cigarettes by individual smokers was carried out as follows: The subject smoked two cigarettes (A and B) simultaneously by taking alternate puffs on each sample. Initially A was smoked through a Cambridge filter and B normally. After three puffs, the system was interchanged, B being smoked through a Cambridge filter and A normally. After three more puffs reversal took place again and so on. By this method the smoker obtained the smoke of two half cigarettes, whereas the smoke of the other two halves was absorbed on the filters. Each subject took 12 puffs per cigarette at a puffing rate of 2/min./cigarette. Smoke exhaled upon each puff was again blown into Cambridge filters (one per cigarette). At the end of the experiment, the four filters (two entry-, two exit-filters) were removed from their holders and submitted to steam distillation as described below. The length of cigarette smoked (or the weight of tobacco burnt) was determined by

<sup>1)</sup> Apparatus constructed by the Research Establishment of the British American Tobacco Co. Ltd., Southampton, England.

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measuring the length of the stub. From the amounts of nicotine and radio-activity recovered from the filters, the percentages of nicotine transferred (nicotine entering the mouth in terms of nicotine in tobacco burnt) and absorption (difference in the amount of nicotine on entry and exit-filters) were calculated. Comparison of the values obtained with the two sets of filters (cigarette A as compared to cigarette B) showed good agreement (standard deviation  $\pm 1\%$ ). The same smoking method was applied for measuring transfer and absorption with regular-brand, non-labelled cigarettes.

Smoking of radio-active cigarettes by rabbits was carried out according to the following technique: A smoking device was attached to the opened trachea of an anaesthetized animal. Smoke was generated by having the rabbit puff cigarettes by its own respiratory activity. By inserting appropriate valves, the air intake was connected to a burning cigarette, whereas the output passed through a Cambridge filter. To prevent excessive smoke condensation, the length of the glass tube (distance cigarette to trachea) was reduced to 8 cm. Since rabbits did not tolerate undiluted smoke, a suitable smoke/air cycle (30 puffs of smoke/30 puffs of air) was introduced. Under these conditions the animals concerned could be kept alive for periods of up to 10 minutes.

#### Physiological methods

Measurements of radio-activity in blood were carried out on rabbits. Animals weighing approximately 3 kg were anaesthetized with Nembutal, and one carotid artery and one jugular vein were cannulated. At time zero, 7 ml of blood (control) was taken from the artery, and the nicotine solution (+ anticoagulant) was injected intravenously. Samples of 7 ml blood were then removed from the artery at different time intervals. Immediately after the last blood sample had been re-

removed the rabbits were killed by air being introduced into the vein. They were dissected without delay, and the organs were frozen by immersion in liquid nitrogen. The same method was applied for analyzing blood and tissues of rabbits subjected to smoking and of those animals receiving nicotine by gastro-intestinal administration.

For urinary elimination experiments, rats and rabbits were housed in metabolism cages, which permitted quantitative collection of urine and faeces. Nicotine was administered to rats by intraperitoneal and rabbits by intravenous injection. To secure an equal and sufficient flow of urine, known amounts of saline were introduced directly into the stomach before and during the experiments. The collected samples of urine and faeces were immediately frozen and stored at  $-15^{\circ}\text{C}$ .

For determining elimination of labelled carbon dioxide and for measuring the balance of radio-activity, rats and rabbits were housed in glass metabolism cages and allowed neither water nor food. Respiratory  $\text{CO}_2$  was swept from the cages by a constant and measured flow of air, and absorbed in 4N sodium hydroxide solution.

Urinary elimination of radio-activity by smokers was followed by collecting the total volume of urine for a period of 30 hours. Subjects were allowed to the same urination schedule in order to have comparable data on their rates of elimination.

#### Chemical and biochemical methods

For determining nicotine and radio-activity representing nicotine in tobacco, on filters and in stubs, the material was submitted to alkaline steam distillation according to the micro-method of Trim (1946). Interfering substances were removed by prior distillation under acid conditions. Labelled nicotine was measured by combustion of an



appropriate sample of distillate, whereas the non-labelled alkaloid was determined by the spectro-photometric procedure of Willis et al. (1956).

Non-labelled nicotine in urine of smokers was determined with a modification of the nephelometric procedure of Mokranjac et al. (1953). This method consists in nicotine distillation from alkaline urine, extraction of the distillate with ether, and precipitation of nicotine with phosphomolybdate in the acid residue after removal of ether by evaporation. Recovery by this method was found to vary between 85 % and 100 %.

Radio-activity representing unchanged nicotine in rabbit blood was extracted by a procedure combining the methods of Wolff et al. (1954) and Hucker et al. (1960): "Liquemized" blood was diluted with saline, and the proteins were precipitated by dropwise addition of 10 % trichloroacetic acid under constant mechanical stirring. An aliquot of the supernatant was alkalinized with 1N NaOH and extracted twice with three times its volume of heptane containing 1.5 % amyl alcohol (heptane purified by successive washings with 1N NaOH, 1N HCl and  $H_2O$ ). The heptane and water phases were separated by centrifugation. The combined heptane fractions were then extracted with 0.1N HCl in a centrifuge tube. After shaking and centrifuging, the organic phase was removed by aspiration. Radio-activity representing nicotine was measured by combustion of the acid phase. Regular checks of this phase by radio-chromatography revealed only nicotine. Recovery of the alkaloid by this procedure varied between 80 % and 85 %.

Chloroform-soluble radio-activity was extracted from tissues (liver, kidneys, lungs, brain, muscle, heart, etc.) by the following procedure: A weighed portion of de-frozen tissue (5 - 20 g) was cut into small pieces and homogenized with twice its quantity of saline in an all-glass Potter Elvehjem homogenizer. Proteins of the homogenate were precipitated by dropwise addition of trichloroacetic acid

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30 % (5 ml/5 g tissue) under constant mechanical stirring. The precipitate and supernatant were separated by centrifuging at 4000 rpm. The former fraction washed twice with small portions of saline (all steps carried out in the cold room). A suitable aliquot (depending on radio-activity) of the combined supernatant and washings was alkalinized (pH 12) with ammonia and extracted in a separatory funnel with three times its volume of chloroform. The chloroform extract was then acidified by adding 5 drops of conc. HCl. After addition of 1 ml of water, the chloroform was completely evaporated under reduced pressure at 50°C. The residual acid was collected and the recipient rinsed several times with small portions of water. The acid and the washings were combined and made up to a small volume (2 - 3 ml). Portions of this aqueous acid fraction were used for combustion and radio-chromatography. Urine of smokers was extracted with chloroform according to the same procedure. Recovery of radio-activity in the final aqueous acid solution after addition of  $C^{14}$ -nicotine to tissue homogenates or to human urine varied between 75 % and 95 %.

Radio-chromatograms of animal urines and extracts of tissues, human urine and blood, or of tobacco and filter distillates were prepared on filter paper Schleicher & Schüll No. 2043a with the descending technique. For routine separation, the n-butanol: ethanol: ammonia: water system of Bowman et al. (1950) was used. To secure reproducible R<sub>F</sub> values and compact spots, the paper was buffered with a borax-boric acid mixture (0.3 M, pH 7.3). Reference spots of nicotine, cotinine, hydroxycotinine and 7-(3-pyridyl)-7-methylaminobutyric acid were revealed by the Koenig reaction according to Dawson et al. (1959). For double checking the identity of unknown radio-active spots, samples were run with the following two solvent systems: (1) n-butanol: water: citric acid (Curry & Powell (1954)) and (2) sec. butanol: formic acid: water (Hausman (1962)). Papers used with acid solvent systems were buffered at pH 4 with 5 % sodium dihydrogen citrate (Curry & Powell (1954)). Reference spots were revealed with a modified Dragendorff reagent (Thies & Reuther (1954)).

#### Radio-chemical methods

The methyl-labelled  $C^{14}$ -nicotine<sup>\*)</sup> (specific activity = 1.45 mc/mg) was stored as the dipicrate. Conversion to the hydrogen tartrate was carried out by passing a picrate solution through a small column of Dowex 2 x 8 (OH<sup>-</sup>). After measuring the amount of radio-active base in the effluent (90 % - 95 %), two moles of d-tartaric acid were added per mole of base, and the aqueous solution was concentrated to the desired volume in a rotary type vacuum evaporator.

For measuring total radio-activity in tissues, body fluids, extracts, residues, distillates, tobacco, etc., appropriate samples were submitted to dry combustion according to the method of Kalberer & Husemann (1961). Carbon dioxide liberated was absorbed in a methanolic ethanolamine solution. An aliquot of this solution was mixed with a liquid scintillation system consisting of toluene, PPO (2,3-diphenyl-oxazole) and POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene). Counting was carried out in a Tri-Carb liquid scintillation spectrometer model 214-X at -5°C according to standard procedures.

Carbon dioxide absorbed in NaOH during respiratory experiments was liberated by excess HCl, driven through a silicagel column by a stream of nitrogen and then trapped in methanolic ethanolamine solution.

Radio-chromatograms were cut into strips and scanned for activity with a Nuclear Chicago Actigraph II connected to a model D-47 glass flow counter. Peaks representing radio-activity were evaluated quantitatively by measuring their surface areas and comparing them with the surfaces of peaks produced by known amounts of  $C^{14}$ .

<sup>\*)</sup> Prepared by Dr K. Decker, University of Freiburg i. Br., Germany.

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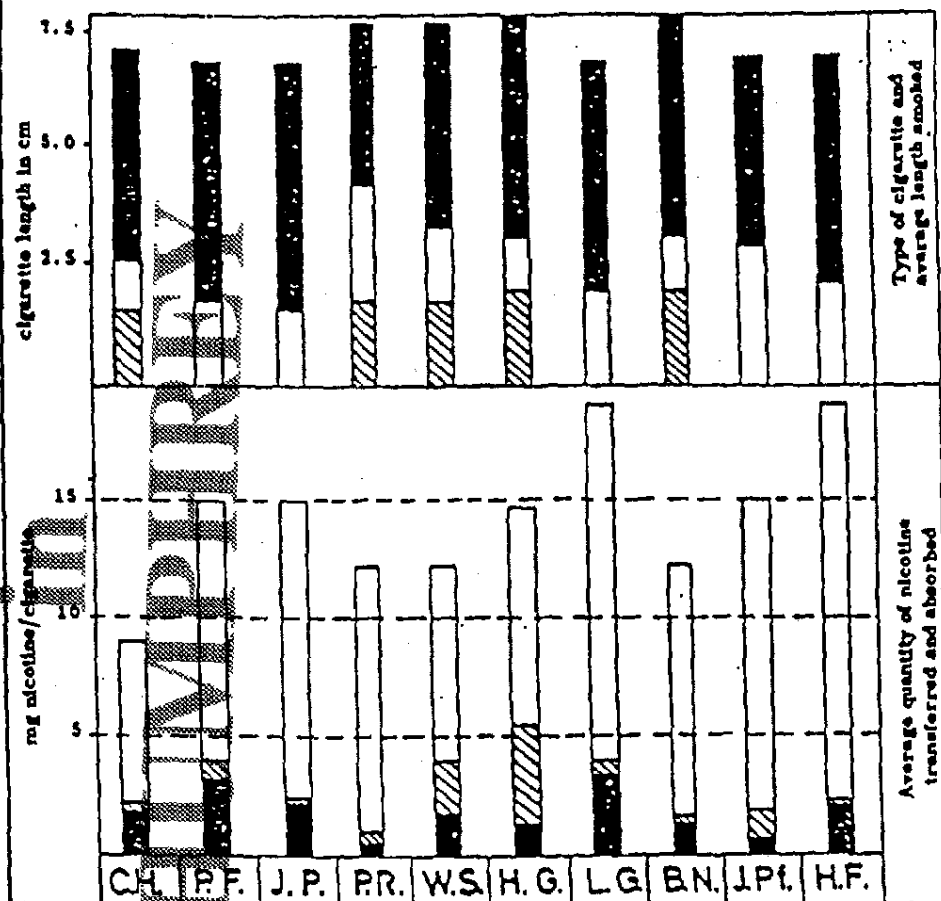
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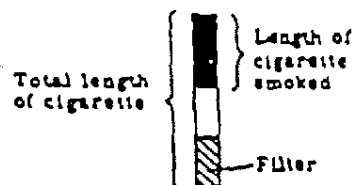
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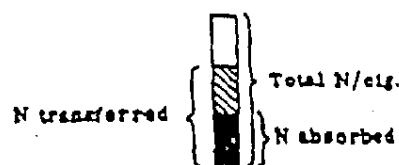


**Fig. 1** Average quantity of nicotine absorbed from one cigarette by individual smokers as compared to their smoking habits.

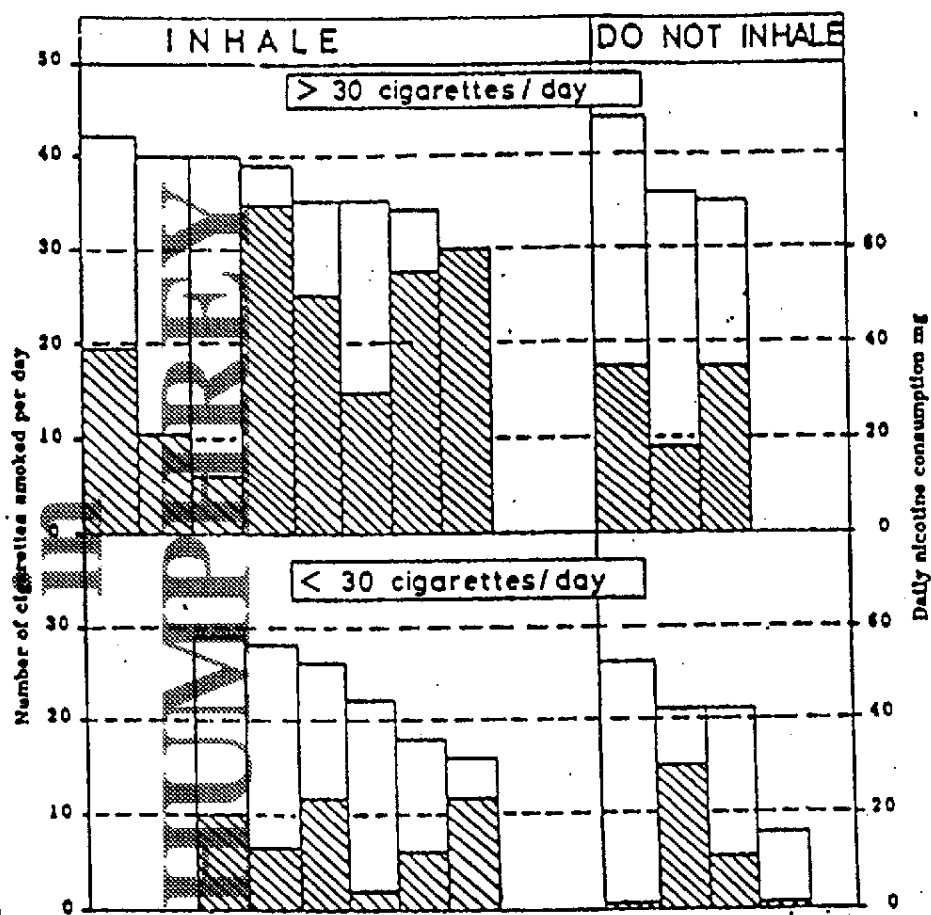
**Above:** Type of cigarette and average length smoked



**Below:** Nicotine present in one cigarette and quantities of N transferred and absorbed







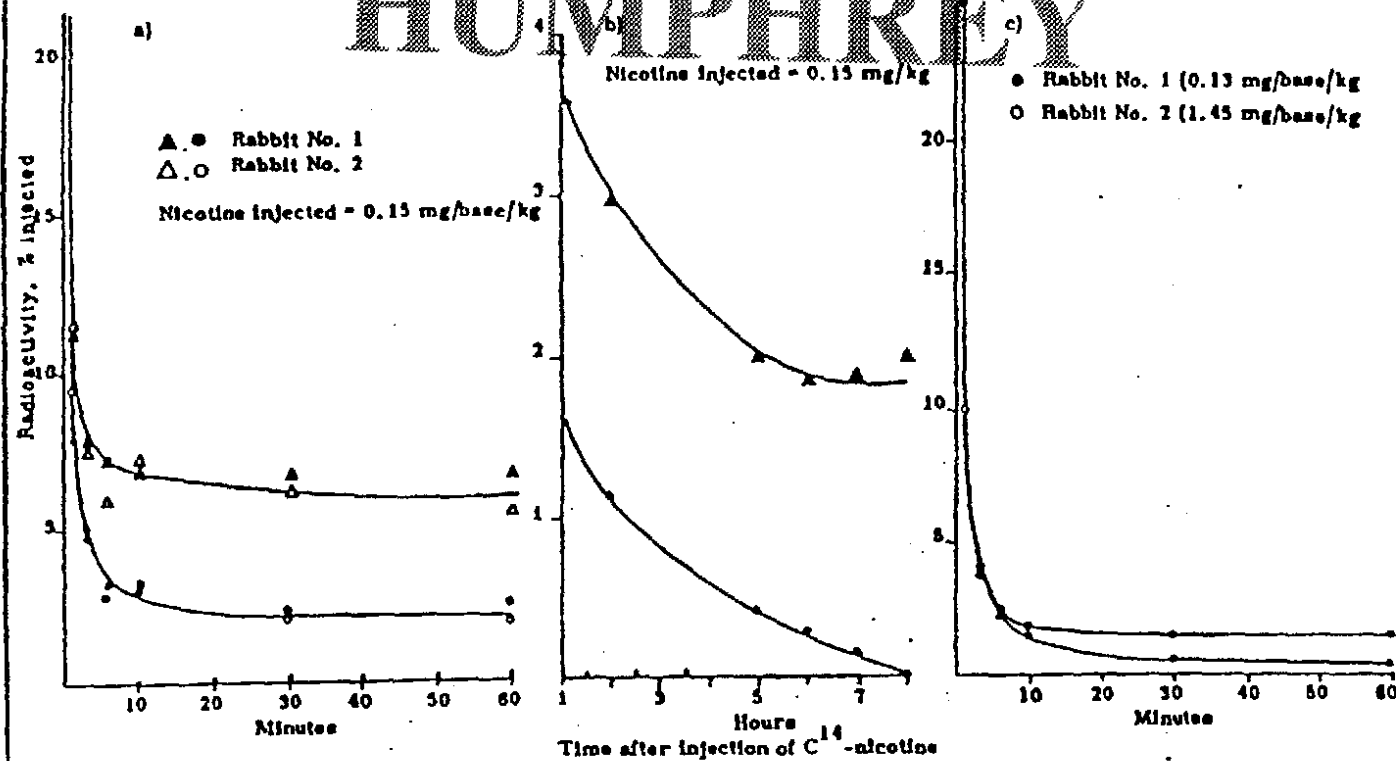
**Fig. 2** Daily nicotine consumption of 21 individual (male) smokers, as determined from average daily quantity of nicotine eliminated in the urine.

□ Number of cigarettes smoked per day  
 ▨ mg nicotine absorbed per day  
 (calculated by multiplying the average quantity of nicotine eliminated in urine by a factor of 10)  
 Nicotine elimination in urine followed for three days.

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Fig. 3 Total radioactivity ( $\Delta$ ,  $\triangle$ ) and radioactivity representing unchanged nicotine ( $\bullet$ ,  $\circ$ ) in rabbit blood as a function of time after intravenous injection of (methyl- $C^{14}$ )-nicotine. Values expressed in % of radioactivity injected.

- a) Short time experiment with two different rabbits      b) Prolonged observation period (1 rabbit)  
c) Short time experiment with two rabbits having received different quantities of nicotine.

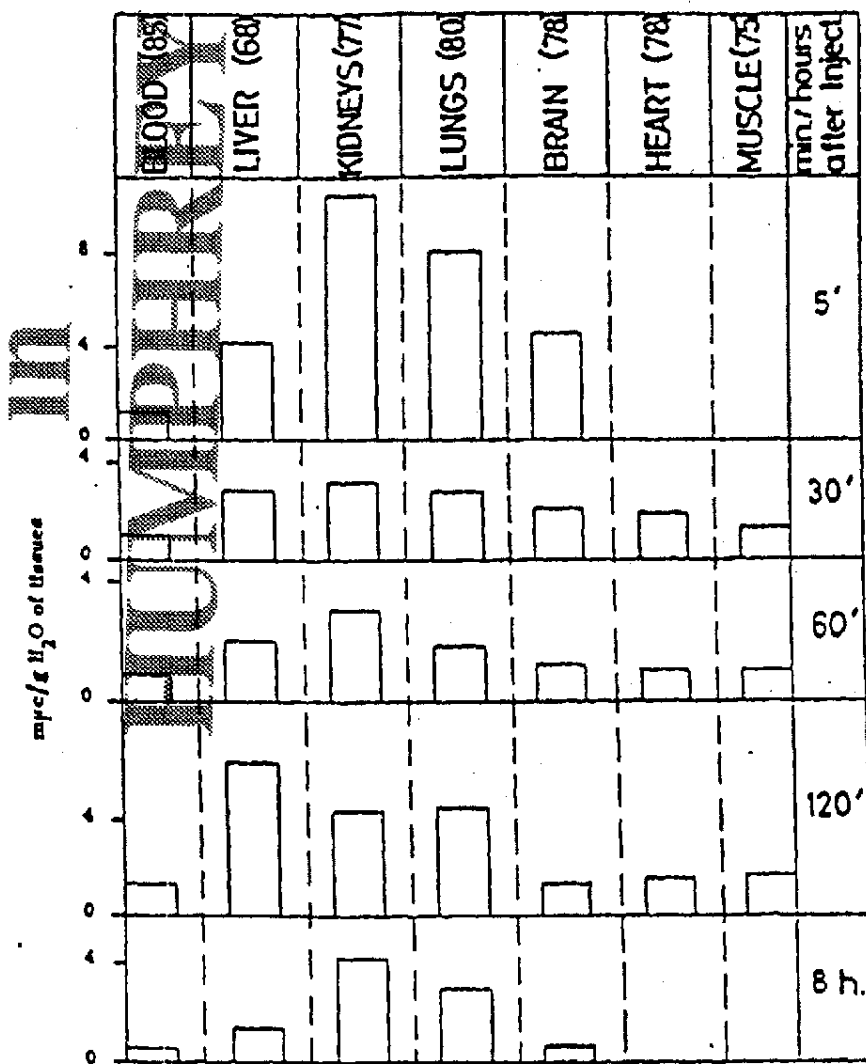


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**Fig. 4** Total radioactivity in rabbit tissues at different time intervals after intravenous injection of (methyl)- $C^{14}$ -nicotine diphosphate.

Radioactivity expressed in  $\text{m}\mu\text{c/g H}_2\text{O}$  of tissues  
 Percentage of  $\text{H}_2\text{O}$  in tissues given in parentheses  
 Values extrapolated for injected quantity of  
 0.15 mg/kg (1.3  $\mu\text{c/kg}$ )  $C^{14}$ -nicotine base



**Fig. 3** Radioactivities representing nicotine (■), cotinine (▨) and hydroxycotinine (□) in various organs of rabbits as a function of time after intravenous injection of  $C^{14}$ -nicotine dihydrate.

Radioactivities measured by scanning of paper strip chromatogrammes and expressed in mpc/g of tissue.

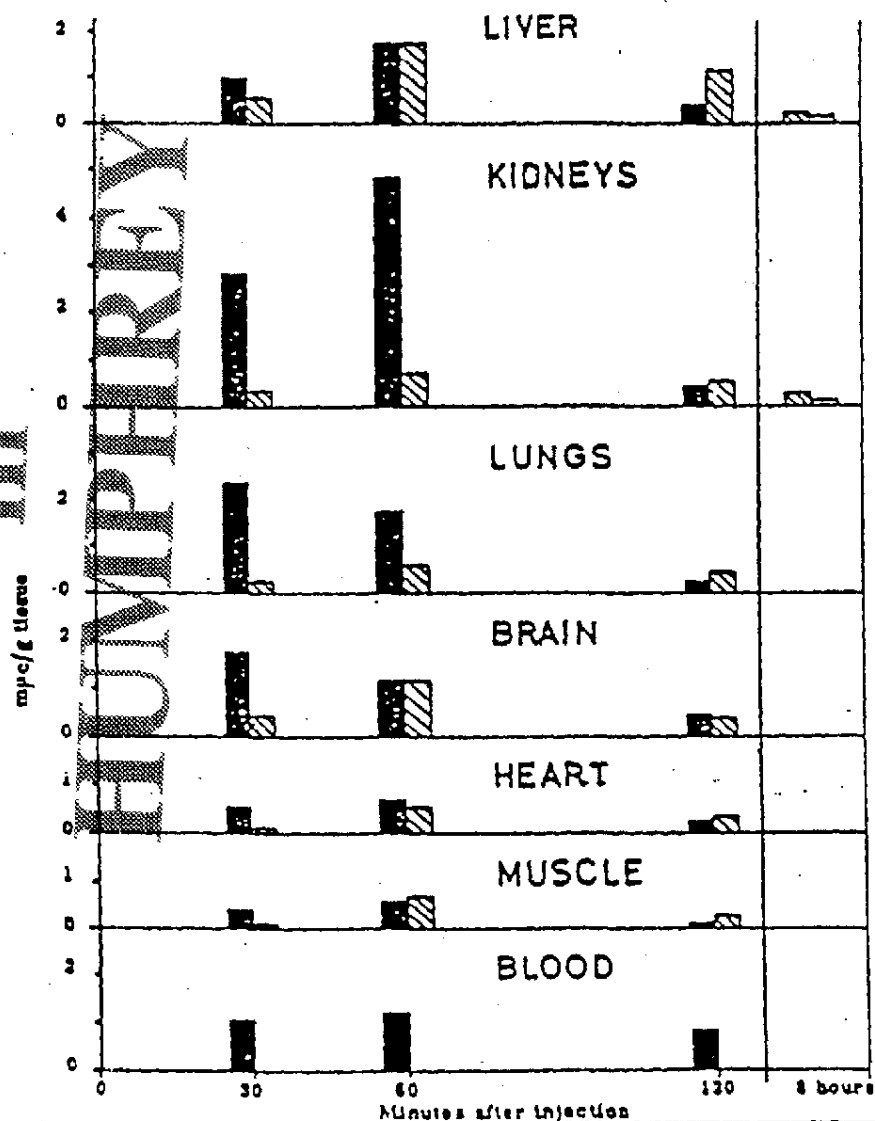


Fig. 6

Radioactivities representing nicotine (■), cotinine (▨) and hydroxycotinine (□) in various organs of rabbits 60 min. after intravenous injection of different concentrations of nicotine (isotope dilution analysis).

Quantities of nicotine injected:

Rabbit A: 0.36 mg  $C^{14}$ -nicotine = 0.13 mg/base/kg

Rabbit B: 0.36 mg  $C^{14}$ -nicotine + 3.85 mg nicotine = 1.43 mg/base/kg

Rabbit C: 0.36 mg  $C^{14}$ -nicotine + 6.35 mg nicotine = 2.30 mg/base/kg

Radioactivities measured by scanning of paper strip chromatogrammes and expressed in mμc/g of tissue.

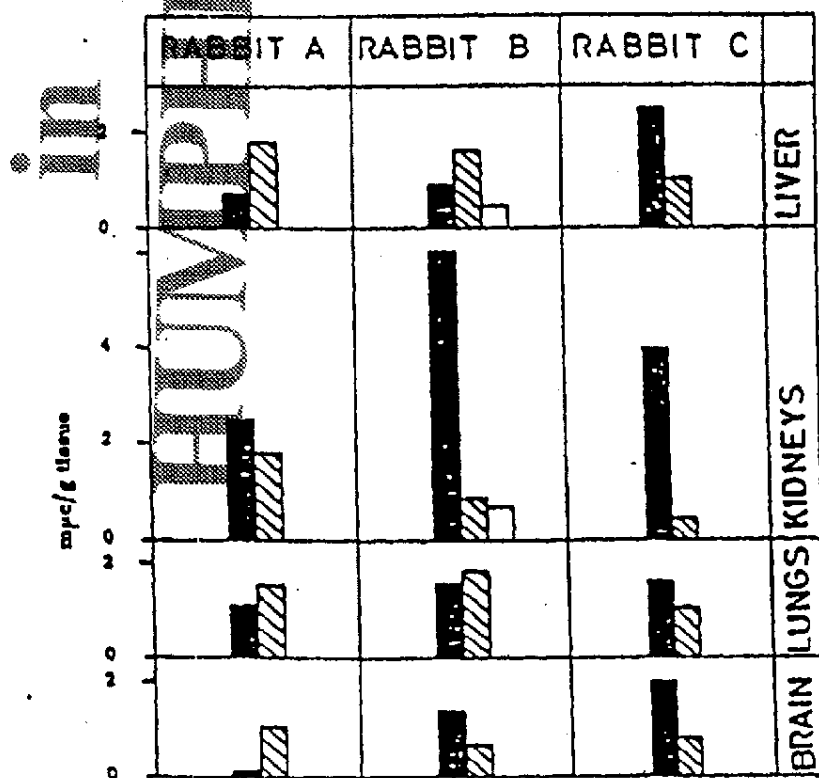
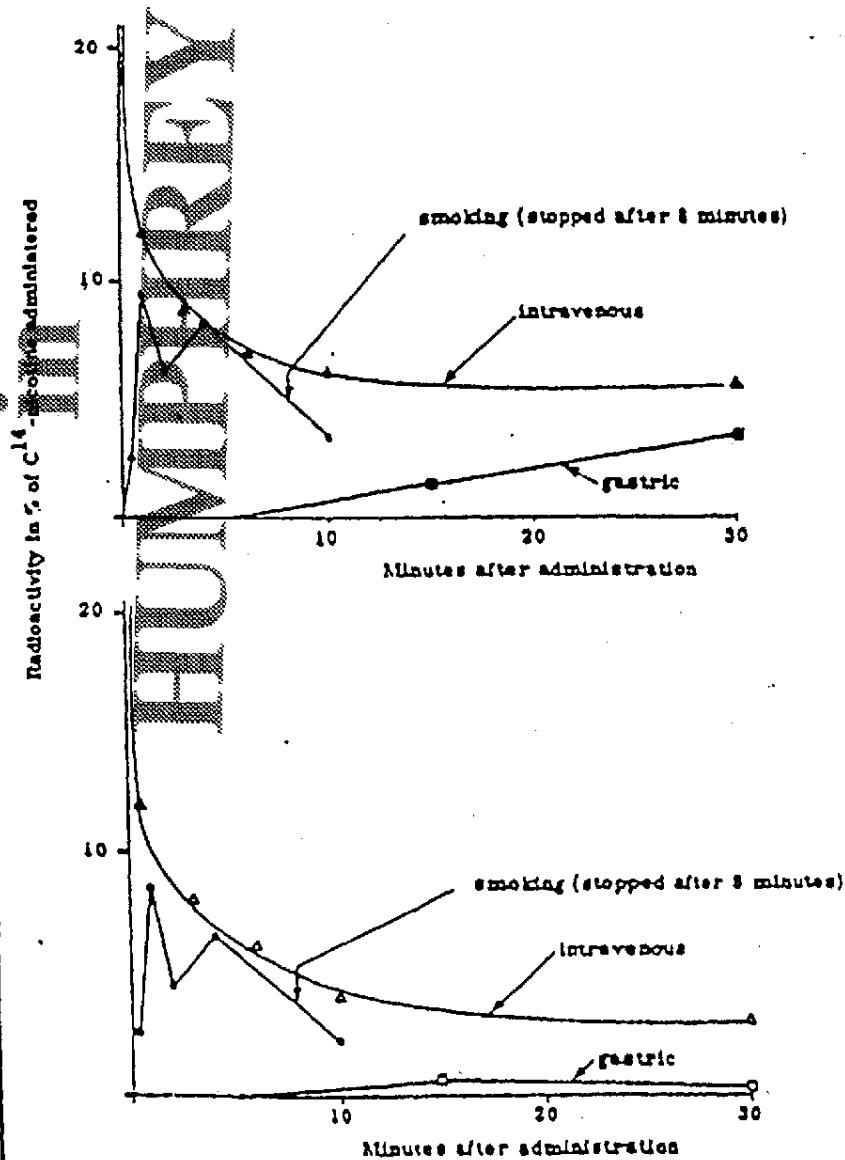


Fig. 7

Total radioactivity ( $\bullet$ ,  $\Delta$ ,  $\square$ ) and radioactivity representing unchanged nicotine ( $\circ$ ,  $\triangle$ ,  $\square$ ) in blood as a function of time after intravenous injection and gastric and pulmonary (smoking) absorption of (methyl)- $C^{14}$ -nicotine.



DU-W2-00046

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**Fig. 3**

Radioactivity representing nicotine (■), cotinine (▨) and hydroxycotinine (□) in various organs of two rabbits after pulmonary absorption of cigarette smoke containing (methyl)-C<sup>14</sup>-nicotine hydrogen tartrate.

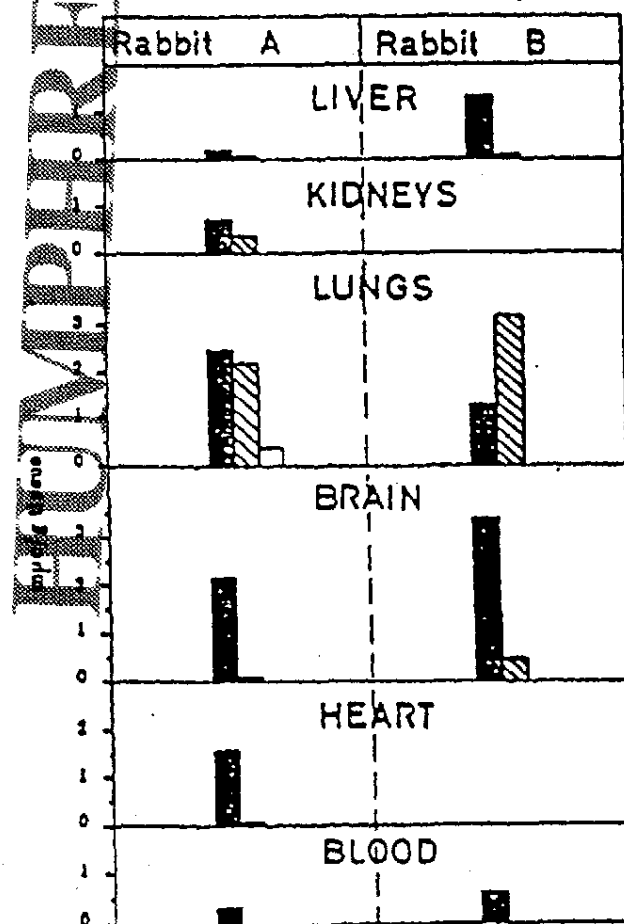
Rabbit A sacrificed 3 minutes after smoking had been stopped

Rabbit B sacrificed 2 minutes after smoking had been stopped

Values expressed in  $\mu\text{c/g}$  of tissue.

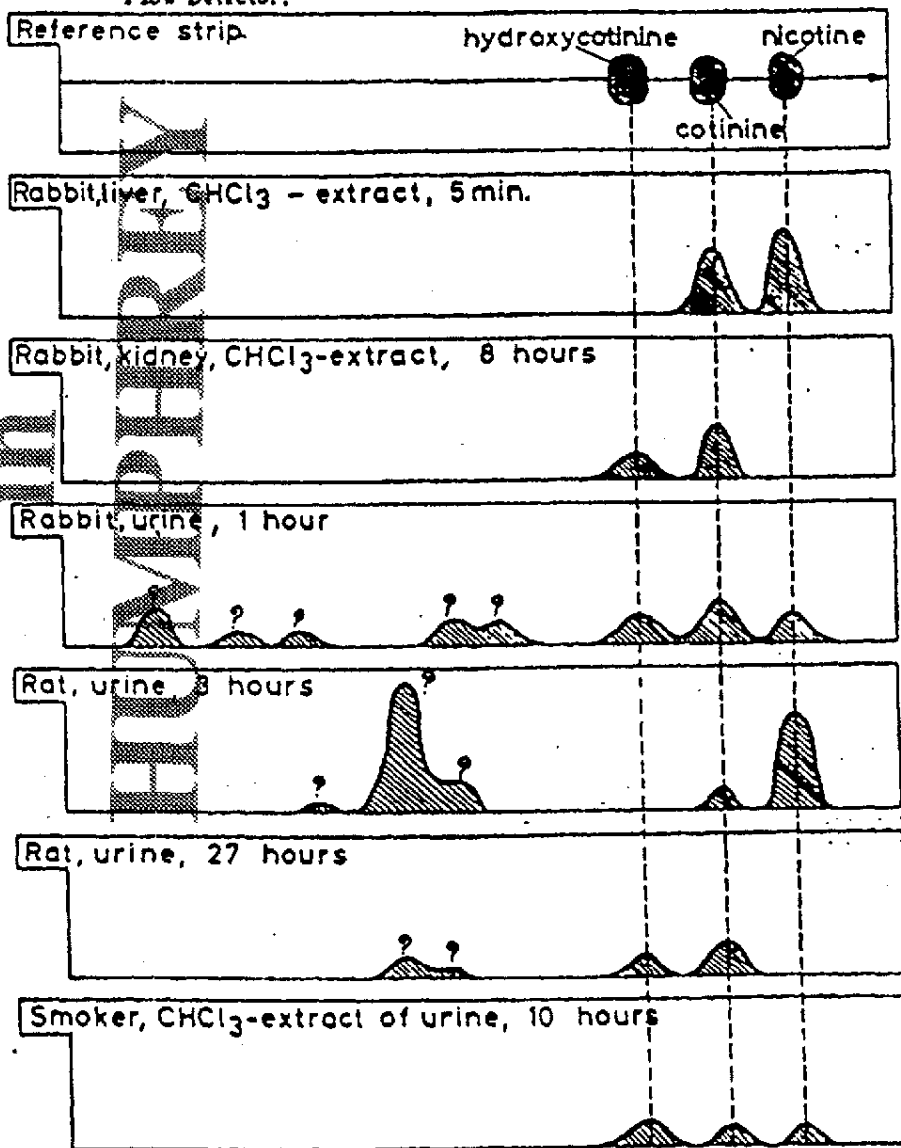
Rabbit A absorbed 3.19  $\mu\text{c}$  or 1.48 mg base

Rabbit B absorbed 3.51  $\mu\text{c}$  or 2.58 mg base



BU-WZ-00047

**Fig. 8** Typical paper-radiochromatograms of tissue extracts and urines of smokers and animals after administration of (methyl)- $C^{14}$ -nicotine. Solvent system: Ammonia water: butanol: ethanol. Scanning of strips with Nuclear Chicago Actigraph II and Model D-47 Gas Flow Detector.





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Fig. 10

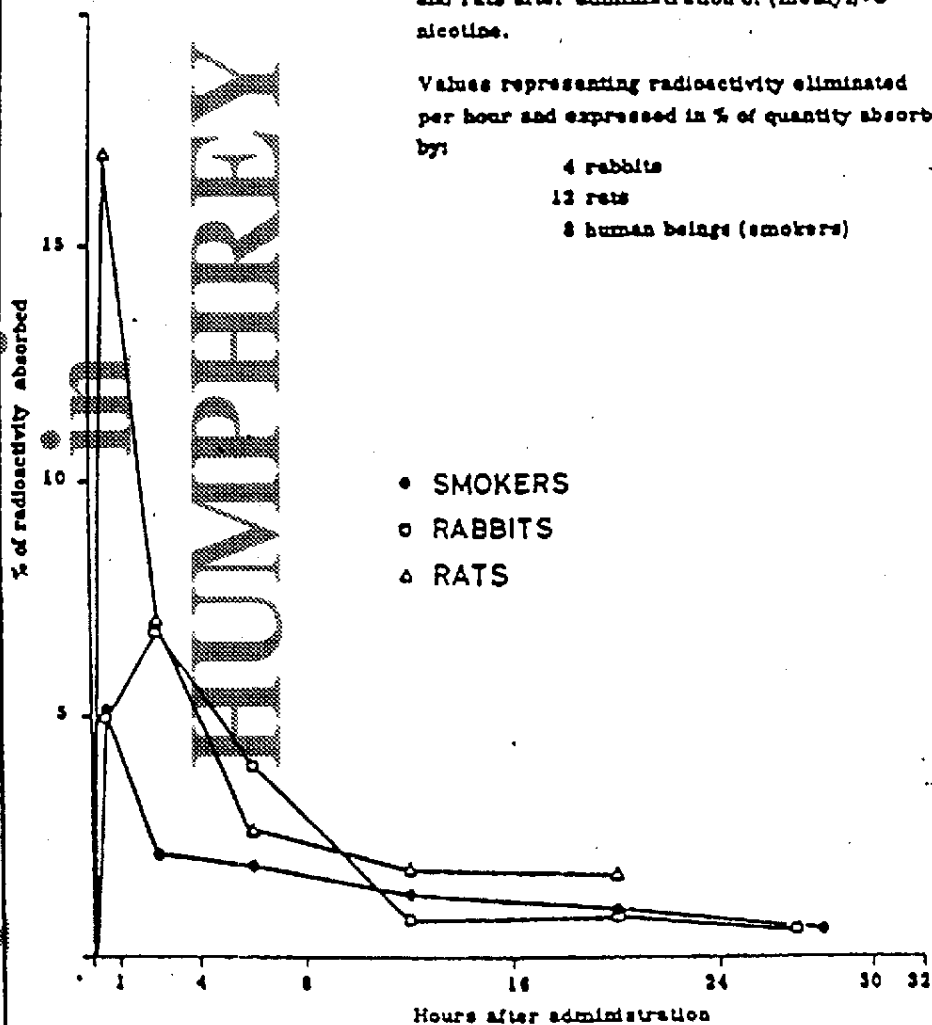
Rate of elimination of total radioactivity in urine of human beings (smokers), rabbits and rats after administration of (methyl)- $C^{14}$ -nicotine.

Values representing radioactivity eliminated per hour and expressed in % of quantity absorbed by:

4 rabbits

12 rats

8 human beings (smokers)



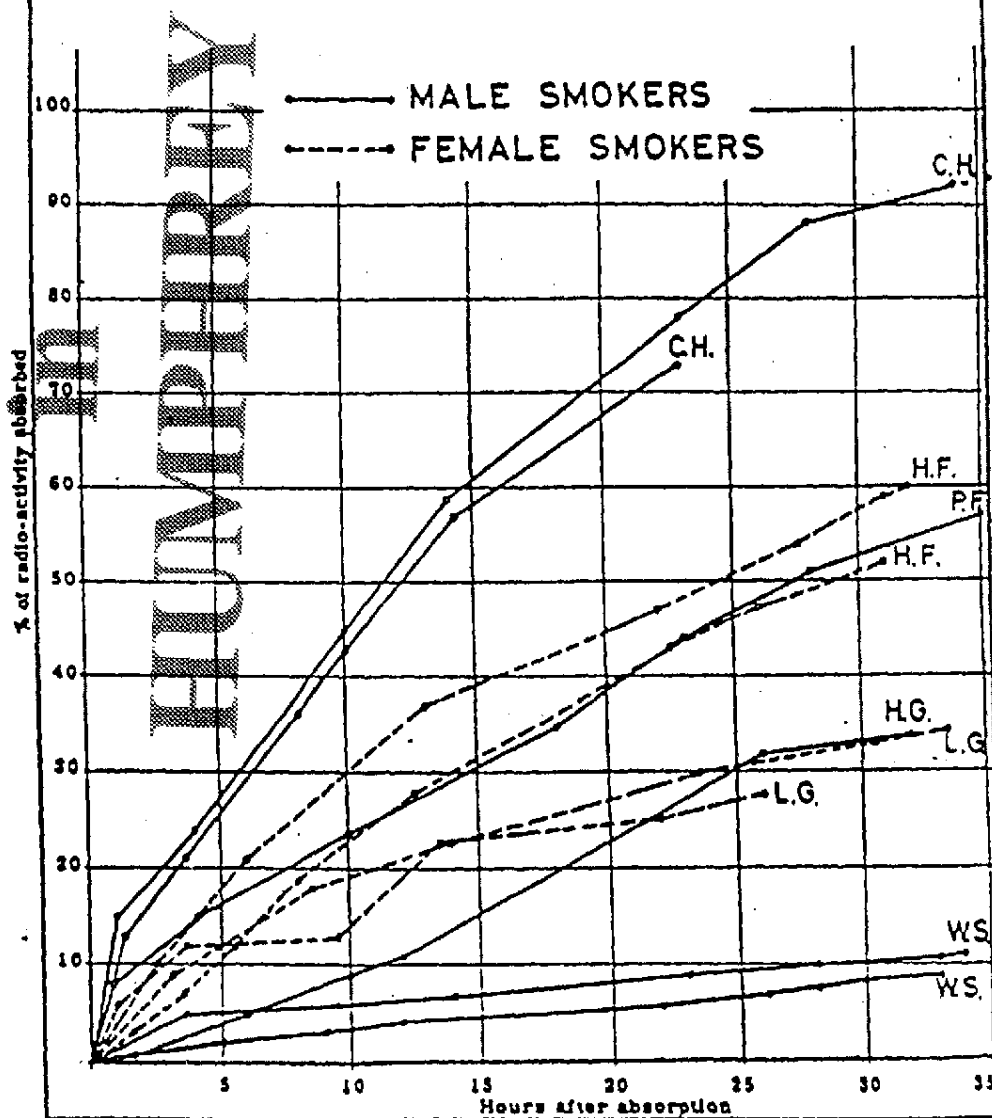
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Fig. 11

Elimination of total radio-activity in urine of six representative smokers as a function of time after smoking of cigarettes fortified with (methyl)- $C^{14}$ -nicotine.

Values expressed in % of radio-activity absorbed.



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8V-V2-00051

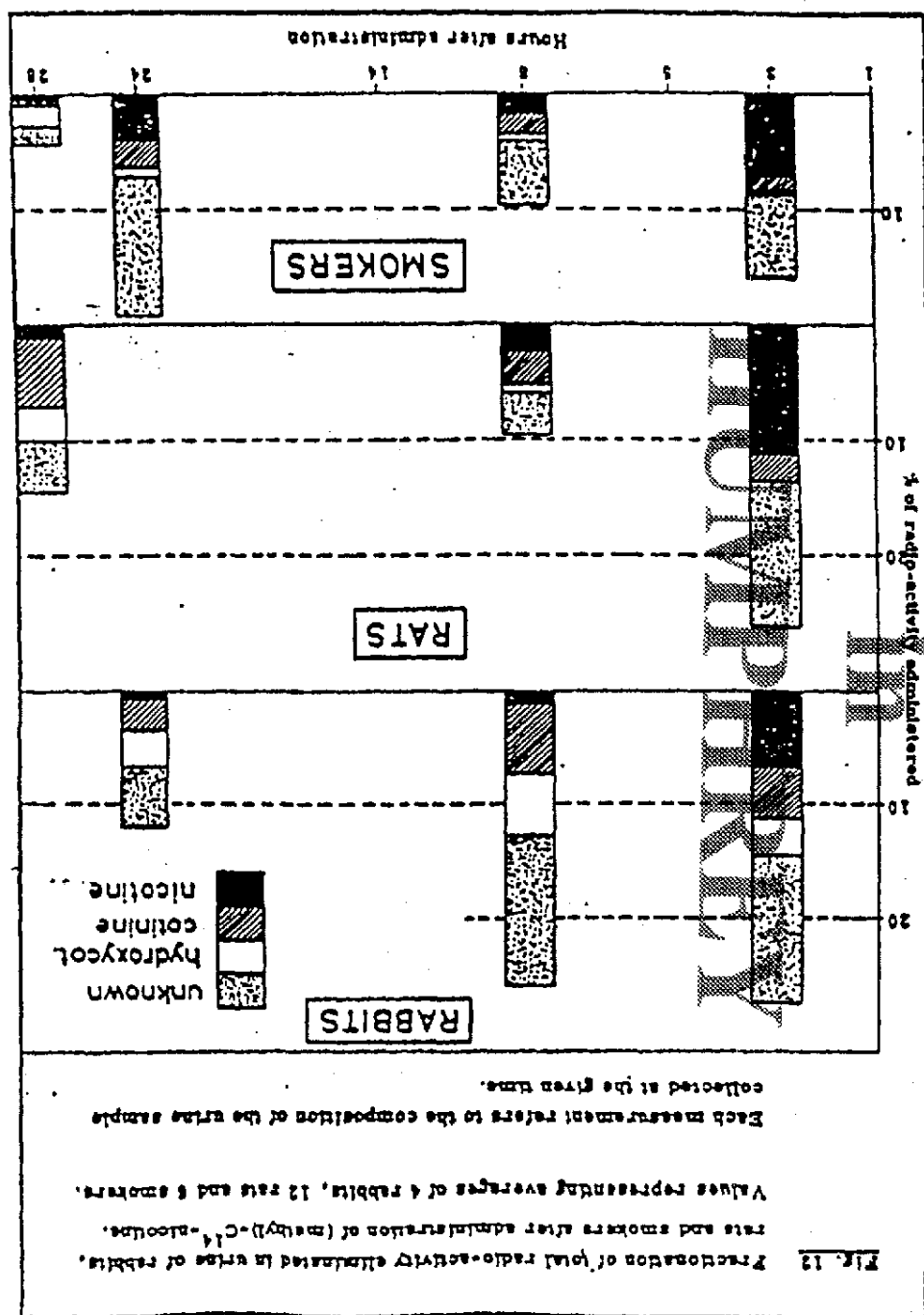
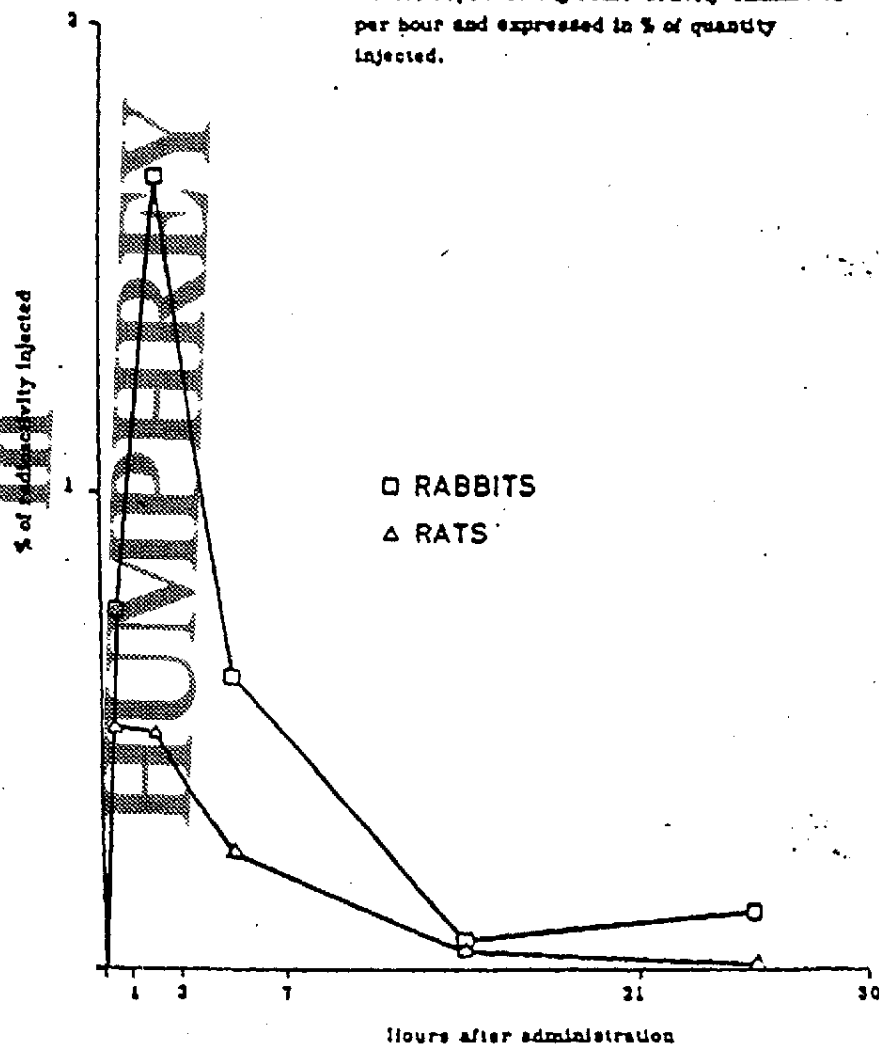
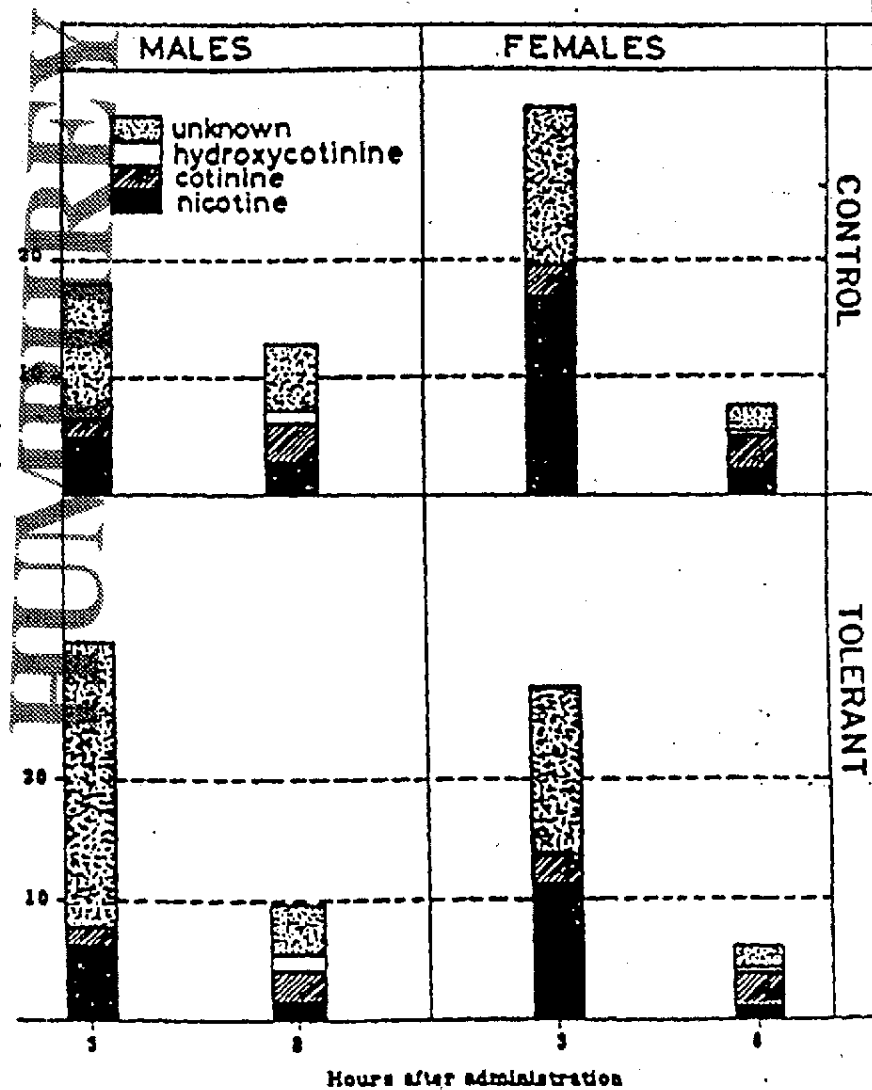


Fig. 13

Rate of elimination of labelled carbon dioxide  
in expiratory air of rabbits and rats after intra-  
venous injection of (methyl)- $C^{14}$ -nicotine.

Values representing radio-activity eliminated  
per hour and expressed in % of quantity  
injected.



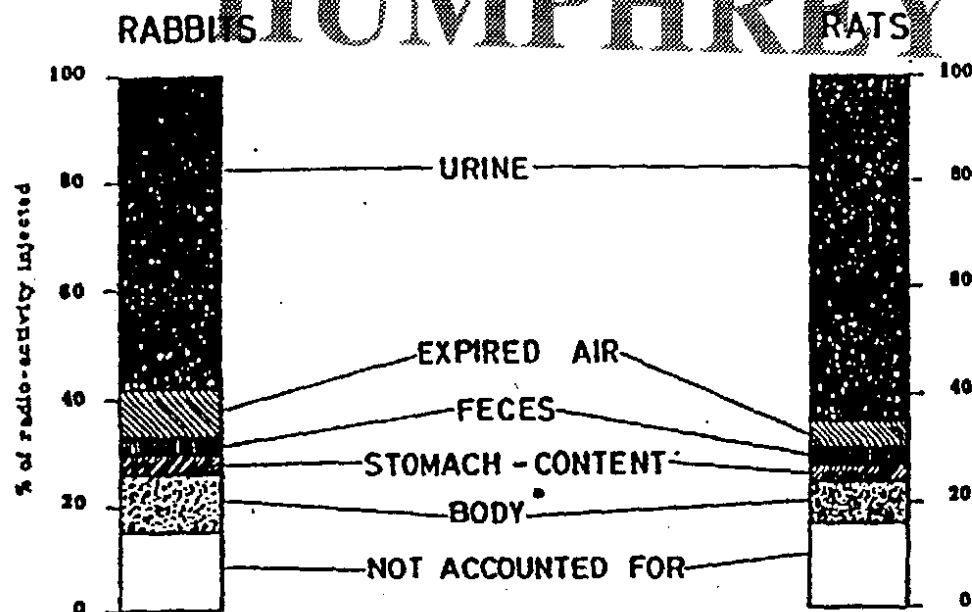


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**Fig. 15** Balance of radio-activity 72 hours after administration of (methyl)-C<sup>14</sup>-nicotine to rats and rabbits.

Values representing averages obtained on two animals of each species.



\* Approximate value, calculated from radio-activity recovered from blood, muscle tissue, adipose tissue and eliminatory organs (liver, kidneys, lungs).

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STATE OF MINDING IN THE BODY (BATTLE REPORT)

This project was carried out with specific objectives and consequently the report suffers somewhat when viewed as a piece of research for publication.

When discussing the publication merits with Dr. Goldhaber, he also felt the same way and made the point that had he been staying on with Battelle he would have carried out further experiments purely to cover the situation. Unfortunately, he will not be able to do so.

Consequently, I suggest that the present report is not completely suitable for publication purposes. However, the work going on with Rand might at a later date enable it to be drafted into two publications.

BW-W2-00055

52259 8749

STATE OF MICHIGAN IN THE BODY (BATTILLE REPORT)

This project was carried out with specific objectives and consequently the report suffers somewhat when viewed as a piece of manuscript for publication.

When discussing the publication merits with Dr. Hilsenrath, he also felt the same way and made the point that had he been staying on with Battelle he would have carried out further experiments purely to cover the situation. Unfortunately, he will not be able to do so.

Consequently, I suggest that the present report is not completely suitable for publication purposes. However, the work going on with Rand might at a later date enable it to be drafted into two publications.

*h*

BU-W2-00056

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COMMUNICATIONS

HROV & WILLIAMSON TOBACCO CORPORATION  
OUTGOING CABLEDATE July 3, 1963TO: MR. MCCORMICKCITY LONDON COUNTRY: (ENGLAND)

PLEASE TO REJECT YOUR TELEX JULY 3 BOYT OF TIRC AGREED TO WITHHOLD DISCLOSURE  
BATTELLE REPORT TO TIRC MEMBERS OR SAB UNTIL FURTHER NOTICE FROM MR. FINCH  
AGREES SUBMISSION BATTELLE OR GRIFFITH DEVELOPMENTS TO SURGEON GENERAL  
UNDESIRABLE AND WE AGREE CONTINUANCE OF BATTELLE WORK USEFUL BUT DISTURBED  
AT ITS IMPLICATIONS RE CARDIOVASCULAR DISORDERS.

WE BELIEVE COMBINATION BATTELLE WORK AND GRIFFITH'S DEVELOPMENTS HAVE  
IMPLICATIONS WHICH INCREASE DESIRABILITY REEVALUATION TIRC AND REASSESSMENT  
FUNDAMENTAL POLICY RE HEALTH. HOPE TO GET OFF COMPREHENSIVE NOTE NEXT WEEK.

YEAMAN

Sent by: ms

cc: Messrs. Finch, Wade & Griffith  
(2 copies to Cable Dept.)

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LONDON

JULY 3, 1963

MR. YEAMAN (TELEX)

S NR 517

YOUR LETTER 20 JUNE. T.R.C. CONSULTANT SCIENTISTS ADVISE IT IS TOO EARLY TO SUBMIT BATTELLE REPORTS TO SURGEON GENERAL'S COMMITTEE BUT WE THINK THEY WILL AGREE THAT CONTINUATION BY BATTELLE OF THIS WORK WOULD BE USEFUL. CHARLES ELLIS CONVINCED OF BENEFICIAL EFFECTS OF NICOTINE BUT AGREES FURTHER INVESTIGATION DESIRABLE BEFORE PUBLICATION. PLEASE INFORM T.I.R.C.

MCCORMICK

ORIGINAL TO MR. YEAMAN

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COPIES TO MESSRS. FINCH, WADE, GRIFFITH

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BRITISH-AMERICAN TOBACCO COMPANY LIMITED

PO Box 42 Westminster House TALLINN, Estonia 100

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Tallinn, Estonia, 100, 100, 100

1st July 1953.

Mr. A. W. H. H. H.  
1500, Main Street,  
Louisville, Ky.

Dear Sir,

On receipt of your letter of 18th June I had a talk with Charles Ellis as a result of which I telephoned you yesterday as follows:-

"YOUR LETTER 28TH JUNE.

"THE CONSULTANT SCIENTISTS ADVISE IT IS TOO EARLY TO SUBMIT BATTELLE REPORTS TO SURGEON GENERAL'S COMMITTEE BUT WE THINK THEY WILL AGREE THAT CONTINUATION BY BATTELLE OF THIS WORK WOULD BE USEFUL. CHARLES ELLIS CONVINCED OF BENEFICIAL EFFECTS OF NICOTINE BUT AGREES FURTHER INVESTIGATION DESIRABLE BEFORE PUBLICATION. PLEASE INFORM T.I.R.C."

Charles' view is that as the situation has now developed it would be wise for B. & W. not to take the initiative in submitting anything to the Surgeon General's Committee but rather wait and hope that the Committee will ask the individual manufacturers for further details of their research work and then, should this happen, it would give B. & W. the opportunity of submitting the Battelle work and the work on the "Avalon" filter. As further work on both has to be done, the work would be immune from charges of criticism, but its disclosure would demonstrate that B. & W. and its associates had adopted a forward-looking positive policy of research.

Yours ever,

Tony

RECORDED

JUL 1 1953

A. T.

P.S. Since dictating this I have received your telex message and we look forward to receiving your comprehensive note.

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General Comment:

The experimental work described in this Battelle report appears to have been more competently done than the work presented in reports entitled HURPO I and HURPO II. Nevertheless the data have been poorly presented with far too much discussion in the Results section and with Methods described partly in the text and more fully in the Appendix. In addition there is no Summary, so that as the document stands it is extremely difficult to assimilate the data presented.

In the introduction the authors say that "the elucidation of the mode of action of nicotine will ultimately depend on biochemical analysis dealing with the behavior of the nicotine molecule on, and its interactions with, the surface of physiologically active, macromolecular cell constituents." This position will no doubt eventually be reached but in the meantime progress in the elucidation of the action of nicotine is more likely to be achieved by measurement of physiological as opposed to chemical effects.

Amounts of nicotine absorbed in cigarette smoking (p. 4 et seq)

The statement on p. 6 that the quantities of nicotine drawn into the mouth by ten smokers ranged from about 1  $\mu$ g. to more than 5  $\mu$ g. per cigarette is in conflict with the results, obtained using mechanical methods of smoking cigarettes, of Ling and Wynn Parry (1949), of 1.1  $\mu$ g. at Harrogate and with results cited by Larsson (1960). The figures of these authors were 0.9  $\mu$ g., 1.1  $\mu$ g. and 1.4  $\mu$ g. per cigarette. In Fig. 1, R.C. smoking filter cigarettes containing 11.5  $\mu$ g. nicotine transferred 5.5  $\mu$ g. (38%), which percentage seems extraordinarily high. Three other smokers (P.Z., K.S. and L.O.) all transferred about 4  $\mu$ g. nicotine on smoking cigarettes containing nicotine in amounts from about 11-19  $\mu$ g. per cigarette. It is difficult to explain how these figures were obtained because there are no details of the actual smoking process. I have asked Dr. Haselbach for some information on the way the 10 subjects of Fig. 1 smoked their cigarettes, in particular some idea of the frequency and volume of draw. I can't help feeling that either the Battelle

Ling and Wynn Parry (1949). Brit. J. Pharmacol., 4, 313-314.  
Larsson (1960). Ann. N.Y. Acad. Sci., 90, 31-35.  
\*This figure was obtained using T.R.C. tobacco smoke condensate, basic fraction, but it was not a fresh sample.

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Filed For  
Identification

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Exhibit D Date 7/30/96  
Case 95-00934-CA

Filed in Evidence

Defendant's

Exhibit 2 Date 8/7/96  
Case 96-00934-CA

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method of assessing transfer and absorption of nicotine (which is an ingenious one and has been used before by Williamson (1950)) is so artificial that it bears little resemblance to the actual smoking of a cigarette.

The absolute quantities of nicotine absorbed per cigarette are said to vary from 0.5 - 3.5 mg., resulting in daily consumptions of nicotine in amounts ranging from 10-50 mg. These high figures should I think be treated with reserve.

The observation that one of the two non-inhalers absorbed only 10% of administered nicotine, whereas 5 of the 8 inhalers absorbed over 80% is in agreement with data quoted by Larson (loc.cit.). Inhalation, particularly deep inhalation, appears to be a very efficient means of absorbing nicotine.

The only other human experiments are illustrated in Figs. 9, 10, 11 and 12 and of these the most interesting are the experiments of Figs. 11 and 12. In Fig. 11 there is seen to be an enormous variation in the percentage and rate of urinary elimination of radioactivity by smokers smoking acetyl-<sup>14</sup>C-nicotine fortified cigarettes. After 36 hours C.M. (an inhaler) had eliminated in the urine nearly 100% of the radioactivity administered. W.S. (a non-inhaler), however, had eliminated only 10% and in fact eliminated measurable quantities of radioactivity for five days. C.M. and W.S. incidentally apparently absorbed similar quantities of nicotine/cigarette. It is suggested, quite reasonably, that these differences are due to variation in absorption, distribution and elimination of nicotine in different subjects, though whether all inhalers metabolize their nicotine differently from non-inhalers is not so certain. It is, however, an interesting possibility.

Animal experiments using radioactive tracer techniques (p.9 et seq.)

As regards the metabolic experiments on rats and rabbits, the results confirm and in some cases extend those of Hanson & Schmitterlow (1962) and McKenna and co-workers (1959 et seq.) and they are therefore likely to be correct. McKenna and co-workers have been largely concerned with isolating and identifying metabolites of nicotine and not specifically with rates of metabolism, as seems to be the emphasis of the present work. In particular the Battelle workers have confirmed the interesting observation of Hanson & Schmitterlow (1962) that nicotine is found in the brain very rapidly after administration by different routes.

Williamson (1950). 1st Tobacco Chemists Conference, Pennsylvania, State University.

Hanson & Schmitterlow (1962). J. Pharmacol., 117, 91-

... strictly quantitative and in this connection the word "significant" is used very loosely. For example on p. 12 appears the statement ".....significant differences in the concentration of unchanged aldehyde were observed for the second (flat) part of the curve" (Fig. 3(c)). Since each curve represents observations on only one rabbit I am not convinced that they are significantly different. There is a bigger difference between the lower curves of Fig. 3(a) and the curve for Rabbit 1 (Fig. 3(c)) than there is between the two curves of Fig. 3(c). Again, I believe the experiments of Fig. 6 do illustrate the point which the authors wish to make (viz that the breakdown and/or excretory mechanisms in various organs become saturated). However, the use of only one animal for each dose of nicotine is inadequate.

#### Discussion (p. 23 et seq.)

The authors tend to be a little dogmatic and there are certain statements in the discussion with which I personally disagree. This, however, is to be expected. On p. 26, the authors deduce from their analysis on radioactivity eliminated in human urines that the "average smoker" breaks down nicotine at a rate about half that determined with rabbits. The observations shown in Fig. 11, however, indicate that there is an extremely wide variation in the elimination of radioactivity so that the phrase "average smoker" has little meaning.

#### Final comments

1. The smoking data are interesting but there are reasons for doubting their accuracy. The average daily consumption of the Battelle smokers was 27 cigarettes (range 15-46); they can therefore all be classified as moderate or heavy smokers. It would have been better to include data shown in the lower half of Fig. 1 for the ten smokers when they were all smoking the same type of cigarette and also to have some data for mild smokers. There is no information on the way the subjects smoked their cigarettes (frequency and volume of draw) and these are obviously important factors.

2. So far as the animal metabolism experiments go, these seem to be acceptable, with certain reservations that have already been made. I have met Dr. Gellatly, who I believe did most of the experimental work, and he seemed to me to be a perfectly honest, reliable and critical worker.

There are, I feel, some results in this report that are of interest and worth publishing, but it is a tedious report to read

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(possibly because the work extended over a prolonged period) and the message or messages the authors wish to convey do not come over at all clearly. The authors themselves admit (p.27) that the present results offer no conclusive evidence for any particular mechanism involved in tolerance to nicotine, nor do they indicate a lead to the phenomenon of addiction. This important problem was, I imagine, the main object of the research.

I.I.I.

21st August, 1963

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STRICTLY PRIVATE & CONFIDENTIAL.

15th May, 1963.

To :- The Chairman.

From :- Sir Charles Ellis.

SMOKING AND HEALTH RESEARCH.

1. Work on the physiological and psychological aspects of smoking has been under investigation by Battelle at Geneva since early 1959, at a total cost of £40,716. The major portion, £16,500, of this has been incurred since September 1st, 1960.

These investigations have a bearing on the "Smoking and Health" problem, and therefore I suggest we should consider whether any of the results should be made available to the Tobacco Research Council.

2. An important part of this work [£19,216] has been concerned with tracing what happens to nicotine in the body when it is absorbed as smoke or injected as a chemical either into the blood stream or under the skin. In each of these cases the results support the generally held view that the physiological effects of smoking arise from the alkaloid nicotine. Important and quite new information has, however, been found out about the extent to which people really inhale, and what differences this makes to the amount of nicotine they absorb. In addition, the speed of response to nicotine and the time the effects last have been investigated. This group of researches has gone under the name of **MAD BUNGER**.

These results are of great interest in helping towards an understanding of the act of smoking and will be much in our minds in developing the new type cigarette, "ARISE". Therefore I suggest we should not disclose this information at the present time.

3. An associated, but reasonably distinct, research named HERPO [£21,500] has been concerned to find out why people smoke and what is the origin of the habit it has on them. A comparison has been made with the effects of the "tranquilliser" drugs, in particular with reserpine. The result is to show that the action of nicotine is quite distinct from that of reserpine and does not have certain undesirable effects of reserpine. A cigarette soothes and enables the smoker to meet calmly a stressful situation because the nicotine stimulates and enhances directly the

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body's natural reaction to stress. This reaction starts in the brain and leads to the release of a succession of chemical substances [hormones] which culminate in the appearance in the blood of corticosteroids and glucose. These substances, for example in a frightened animal, enable it to release a burst of muscular energy to escape from danger. In man this reaction is more sophisticated and provides for reaction to mental stress and helps mental response. The present-day human reaction to stress is in most people somewhat halting, and many would find it difficult to stand up to the pace and impacts of modern environment without some external assistance. The cigarette, by means of its nicotine, does this by stimulating and enhancing the body's natural reaction. This action is quite distinct from that of tranquillisers or sedatives which merely deaden antagonistic reactions.

These researches have, in addition, shown that the corticosteroids, which are released in the blood by the action of nicotine and produce the above effects, also act directly against obesity. They do this by a three-stage process. Firstly, fat is mobilised from its deposits and brought into the blood stream; secondly, its metabolism and disposal is accelerated and, thirdly, appetite is reduced.

There is a subsidiary anti-diuretic effect, already well-known but which has been verified in the present experiments for the sake of completeness. It does not seem to be important either positively or negatively.

Reserpine, the well-known tranquilliser, blocks all pituitary functions and an important part of this work has been to show that, on the contrary, nicotine, in the doses that can be obtained by smoking, has no effect on, that is, does not block the -

- a] Gonadotrophin releasing factors,
- b] Thyrotrophin releasing factors, -
- c] Somatotrophin releasing factor.

On the other hand, it must be mentioned that Burn has shown nicotine releases nor-adrenaline, which is a vasoconstrictor, and increases blood pressure. However no one, except the fanatics, have claimed this to be a danger, and I understand the majority opinion is now that smoking is not significant in the cardiac-vascular problem.

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3. The people who oppose smoking do so on the grounds that it is an important contributory cause of lung cancer and that it is just a habit with nothing to be said for it other than that it is pleasurable. Once given up it would soon be forgotten. It is my opinion that the results I have described show this view to be untenable. Nicotine is a wonderfully beneficent drug which does not, like morphine, sleeping pills or even dextroin, lead to cumulative addiction. People keep to their smoking habits over years. If nicotine were not known its discovery would be claimed as one of the great medical advances of the day.

Its absorption by smoking depends on a simple but subtle technique of releasing the nicotine by a method closely under the control of the smoker. It is this which has built up the psychological aspects of smoking which are so strong and so important, but these only arise as a result of the physiological effects being associated by a memory process with a cigarette. Thus a smoker already feels calmed as he takes a cigarette from his case; indeed, it can be shown that there is a physiological response based on subconscious memory even before the first puff is taken.

4. The important result of this research HBP0 is that it gives an experimental basis for believing that the tobacco industry is carrying out an essential and valuable service for the public. It is my considered opinion that the tobacco industry has no reason to allow itself to be pushed onto the defensive; on the contrary, it is justified in taking a positive position that it is providing a product of which it is proud and from which the public benefits. The industry could safely admit that there may be some undesirable features in the products of combustion of the tobacco and paper, but it is vigorously carrying out research to find methods of removing these. It is also recognised that some "cheasy" or bronchitic people would be well advised not to smoke, but here again important research is supported by I.R.C. to define this group more precisely.

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5. It will be appreciated that the possibility of taking this attitude depends on the results of our research HBP0, and it is for this reason that I consider it would be wise to disclose them to I.R.C. In the first instance, the Battelle experiments would be examined by the Technical Sub-Committee and also by the I.R.C. medical experts, such as Dr. Day, Dr. Ambrose, and their consultant Professor Burn. If the report survives their critical appraisal then, and only then, will I.R.C. face

the policy decision whether it would be in the best interests of the industry -

- a] to keep this material confidential but allow it to modify their attitude,
- or
- b] to keep the material confidential but show it to E.I.R.C. and the Forschungstelle in Germany,
- or
- c] additionally to show it to our friends in medical circles, such as Hadow, Dodds, Marrian, Rose, etc.

The question of publication in a scientific journal scarcely arises at this stage since the results have not been written up for that purpose.

6.

Rather special consideration is required to decide when and how the HIFPO reports should be shown to Louisville, Montreal, Hamburg and Sydney. While it is very much a matter of Group research and as such should be disclosed to them before being shown to any outside body, I should have thought in this case the situation would best be met by a confidential letter disclosing the policy issue involved and pointing out that there was little point in sending over the technical papers until these had survived the critical appraisal of our own experts in this country.

7.

I recommend the Committee should authorize that the following reports should be sent to E.I.R.C. -

Final Report on HIFPO I, dated January 1962.

Report No. 1 on HIFPO II, dated June 1962.

Final Report on HIFPO II, dated May 1963.

I further recommend that members of the E.I.R.C. Research Group should be informed in general terms of the Committee's decision.

*Charles E. Egan*

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\* Copy of these three reports sent to the Chairman.

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NOTE FOR MR. CUTCHINS

On 4th June Sir Charles Ellis sent to you copies of reports of research which B. A. T. had sponsored at the Battelle Research Institute in Geneva showing the beneficial effects of nicotine on the smoker. B. A. T. decided to make this research available to the T. R. C. here and it is being evaluated by T. R. C.'s outside medical experts. Preliminary reports indicate that these experts think the Battelle work to be a sound piece of research. It was always contemplated that if the reports stood up scientifically it might be desirable to get them submitted to the U. S. Surgeon General's Committee.

Todd, of T. R. C., is to-day sending copies to T. I. R. C. with a request that they consider whether it would help the U. S. industry for these reports to be passed on to the Surgeon General's Committee.

I thought you should have this information in case you or any of your colleagues in Louisville might for any reason think this course of action inadvisable.

Could you please let me know as soon as you get back what your views are.

19th June 1963.

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# SMOKING *and* HEALTH

REPORT OF THE ADVISORY COMMITTEE  
TO THE SURGEON GENERAL  
OF THE PUBLIC HEALTH SERVICE

U.S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE  
PUBLISHED BY THE U.S. GOVERNMENT PRINTING OFFICE

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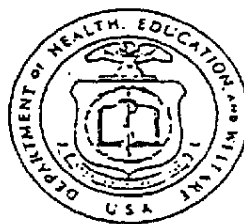
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# SMOKING *and* HEALTH

REPORT OF THE ADVISORY COMMITTEE  
TO THE SURGEON GENERAL  
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U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE  
Public Health Service

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THE SURGEON GENERAL'S ADVISORY  
COMMITTEE ON SMOKING  
AND HEALTH

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Walter J. Burdette, M.D., Ph. D.  
William G. Cochran, M.A.  
Emmanuel Farber, M.D., Ph. D.  
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Since the turn of the century, interest in the effects of smoking, however, has a broad manifest: within this has been undertaken since.

Few medical questions have been the subject of scientific debate than that of smoking and health. Answers must be found.

As the principal Federal American people, the responsibility for seeking seemed necessary to a scientific evidence and of the people of the assessed the then available findings known to the of the evidence and its relationship of cigarette consumption data has accumulated.

Accordingly, I appreciate the scientific disciplines, and, if possible, to relate between smoking and health and evaluation are conducted.

I pledge that the Federal thorough review of the and necessary. I am confident agencies will do the same.

The Committee's assignments and the press duties has been great. The technical report requires professional and technical assistance. The Committee's task has required the highest order of the Committee. The many members of the staff, but for the Nation as a whole.

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## Chapter 2

### CONDUCT OF THE STUDY

The work of the Surgeon General's Advisory Committee on Smoking and Health was undertaken, organized, and pursued with independence, a sense of responsibility, and with full appreciation of the national importance of the task. The Committee's constant desire was to carry out in its own way, with the best obtainable advice and cooperation from experts outside its membership, a thorough and objective review and evaluation of available information about the effects of the use of various forms of tobacco upon the health of human beings. It desired that the Report of its studies and judgments should be unquestionably the product of its labors and its authorship. With an enormous amount of assistance from 135 consultants, from members and associates of the supporting staff, and from several organizations and institutions, the Committee feels that a document of adequate scope, integrity, and objectivity has been produced. It is emphasized, however, that the content and judgments of the Report are the sole responsibility of the Committee.

At the outset, the Surgeon General emphasized his respect for the freedom of the Committee to proceed with the study and to report as it saw fit, and he pledged all support possible from the United States Public Health Service. The Service represented chiefly by his office, the National Institutes of Health, the National Library of Medicine, the Bureau of State Services, and the National Center for Health Statistics, furnished the able and devoted personnel that constituted the staff at the Committee's headquarters in Washington, and provided an extraordinary variety and volume of supplies, facilities and equipment. In addition, the necessary financial support was made available by the Service.

It is the purpose of this section to present an outline of the important features of the manner in which the Committee conducted its study and prepared this Report. A retrospective outline of procedures and events tends to convey an impression of orderliness that did not pertain at all times. A plan was formulated at the first meeting of the Committee in November 1950, but it was found to be modified from time to time as new lines of inquiry and unanticipated explorations. At first an encyclopedic approach was considered, to deal with all aspects of the use of tobacco and the resulting effects, with important aspects of air pollution, and all pertinent characteristics of the external and internal environments and make-up of human beings. It was soon found to be impracticable to attempt to do all of this in any reasonable length of time, and certainly not under the urgencies of the existing situation. The final plan was to give particular attention to the cores of problems of the relationship of uses of tobacco, especially the smoking of cigarettes, to the health of men and women, primarily in the United States.

to deal with the material from both a general viewpoint and on the basis of disease categories.

As may be seen in a glance at the Table of Contents of this Report, the main topical divisions of the study were:

- Tobacco and tobacco smoke, chemical and physical characteristics (Chapter 6).
- Nicotine, pharmacology and toxicology (Chapter 7).
- Mortality, general and specific, according to age, sex, disease, and smoking habits, and other factors (Chapter 8).
- Cancer of the lungs and other organs: carcinogenesis: pathology, and epidemiology (Chapter 9).
- Non-neoplastic diseases of the respiratory tract, particularly chronic bronchitis and emphysema, with some consideration of the effects of air pollution (Chapter 10).
- Cardiovascular diseases, particularly coronary artery diseases (Chapter 11).
- Other conditions, a miscellany including gastric and duodenal ulcer, perinatal disorders, tobacco amblyopia, accidents (Chapter 12).
- Characterization of the tobacco habit and beneficial effects of tobacco (Chapter 13).
- Psycho-social aspects of smoking (Chapter 14).
- Morphological constitution of smokers (Chapter 15).

As the primary duty of the Committee was to assess information about smoking and health, a major general requirement was that of making the information available. That requirement was met in three ways. The first and most important was the bibliographic service provided by the National Library of Medicine. As the annotated monograph by Larson, Haag, and Silvette—compiled from more than 6,000 articles published in some 1,200 journals up to and largely into 1959—was available as a basic reference source, the National Library of Medicine was requested to compile a bibliography (by author and by subject) covering the world literature from 1953 to the present. In compliance with this request, the National Library of Medicine furnished the Committee bibliographies containing approximately 1100 titles. Consequently, the Committee staff was housed in the National Library of Medicine on the grounds of the National Institutes of Health, and through this location had ready access to books and periodicals, as well as to scientists working in its field of interests. Modern apparatus for photo-reproduction of articles was used constantly to provide copies needed for study by members of the Committee. In addition, the members drew upon the libraries and bibliographic services of those institutions in which they held academic positions. A considerable volume of copies of reports and a number of special articles were received from a variety of additional sources.

All of the major companies manufacturing cigarettes and other tobacco products were invited to submit statements and any information pertinent to the inquiry. The replies which were received were taken into consideration by the Committee.

Through a system of contracts with individuals competent in certain fields, special reports were prepared for the use of the Committee. Through these

sources much valuable information was made available to the Committee.

In addition to the special conferences, seminar-like meetings were made available to the Committee. A considerable amount of well-informed

To deal in depth and discretion at its first meeting for membership. These subcommittee reports, analysis, and evaluation reports, discussions at conference reprogrammed and carried on. These will be acknowledged in formulations of conclusions which were submitted to the full Committee.

At the beginning, and until executive session, it had the services from other Federal or following agencies: Executive Federal Trade Commission, Department of Culture, and the Food and Drug Administration. Through written communication, they provided information.

There were an uncounted number of lesser gatherings. Between November and December 1961, the Committee held nine sessions in Washington or Bethesda. The main critical scrutiny of conclusions and editing of this comprehensive

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sources much valuable information was obtained; some of it new and hitherto unpublished.

In addition to the special reports prepared under contracts, many conferences, seminar-like meetings, consultations, visits and correspondence made available to the Committee a large amount of material and a considerable amount of well-informed and well-reasoned opinion and advice.

To deal in depth and discrimination with the topics listed above, the Committee at its first meeting formed subcommittees with much overlapping in membership. These subcommittees were the main forces engaged in collection, analysis, and evaluation of data from published reports, contractual reports, discussions at conferences, and from some new prospective studies reprogrammed and carried out generously at the request of the Committee. These will be acknowledged more fully elsewhere in this Report. The first formulations of conclusions were made by these subcommittees, and these were submitted to the full Committee for revision and adoption after debate.

At the beginning, and until the Committee began to meet routinely in executive session, it had the advantage of attendance at its meetings of observers from other Federal agencies. There were representatives from the following agencies: Executive Office of the President of the United States, Federal Trade Commission, Department of Commerce, Department of Agriculture, and the Food and Drug Administration. Serving as more than observers and reporters to their agencies, when they were present or by written communication, they supplied the Committee with much useful information.

There were an uncounted number of meetings of subcommittees and other lesser gatherings. Between November 1962 and December 1963, the full Committee held nine sessions each lasting from two to four days in Washington, D.C. The main matters considered at the meetings in October, November, and December 1963 were the review and revision of chapters, critical scrutiny of conclusions, and the innumerable details of the composition and editing of this comprehensive Report.

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### Project II

The working of this project is extremely vague. The first thing to ascertain is whether nicotine is affecting the hypothalamo-pituitary system by releasing adrenaline from the adrenal medulla. The object of the experiments proposed (and Battelle have not yet done any work on these lines) is to demonstrate release of corticosteroids by radioactive tracer techniques. Such techniques may be suitable for animals, but will not be suitable for experiments on human beings. We are proposing to measure corticosteroids at Harrogate fluorometrically, which method will be applicable to animals and human beings. This work closely parallels our own and some of that of Professor Ruttle. I believe Battelle might be capable of producing some data more quickly than we shall but this is not a race and it is the quality of the work that matters.

### Project III

These experiments are far too premature for consideration at the moment and in any case Battelle have shown themselves to be quite incompetent to carry out stereotactic techniques.

Now that the T.R.C. has its own Pharmacological Laboratories it should, as a matter of policy, undertake its own pharmacological research, since pharmacology is a subject which does not readily lend itself to contract research. At the moment the size and scope of the Pharmacological Laboratories are adequate for the programme of research we are following. I personally do not think that T.R.C. should sponsor any research at Battelle: even Project I would not I think turn out to be as cut and dried as the Battelle workers seem to think and the estimate of £7,000 to £9,000 given to me by Dr. Geissbühler for carrying out these experiments seems extremely high, considering all the experimental methods have already been worked out. I do not think this would be money well spent.

A.K.A.  
23rd August, 1963.

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VISIT TO BATTELLE INSTITUTE, GENEVA, 8th & 9th AUGUST, 1963.

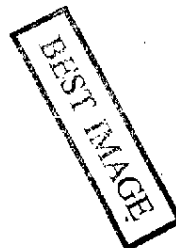
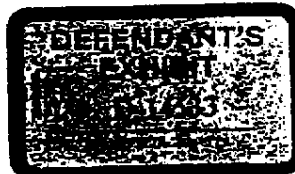
The object of my visit to Geneva was to meet Drs. Haselbach, Libet and Geissbühler (the authors of the four Battelle reports), to discuss the ambiguities and omissions in their reports and to see if Battelle could usefully supplement the pharmacological and biochemical research we shall be doing at Harrogate.

Most of the experimental work of the HIFPO reports was done by Dr. Libet, who is not a pharmacologist, and in consequence she was attempting to do work for which she was not qualified. Neither Professor Burn nor myself are impressed with the HIFPO work. The work of the fourth report "The fate of nicotine in the body" appears to be much sounder as Drs. Geissbühler and Haselbach are biochemists. Even this report, however, is poorly presented though I believe there is some good work in it. It should be remembered that Battelle undertakes many research projects, so that no one project is ever given their undivided attention for any length of time. I believe all the present work suffers from this part-time approach. I spent a couple of days with Dr. Haselbach and his colleagues and got the impression that if they were given a very short term and precise project, that required little or no supervision, they would do it competently. A project like "The fate of nicotine in the body" offers far too much scope and is apt to go rambling on. After much consideration I do not consider we have any problems which fall into this category that we cannot tackle more competently ourselves.

Attached to this visit report is a programme of research to follow up findings of project HIFPO and "The fate of nicotine in the body" suggested by Battelle to the British-American Tobacco Company. I took the opportunity to find out more precisely what the Battelle workers were intending to do.

Project I

The work envisaged would require the use of randomly C<sup>14</sup>-labelled nicotine in preference to the methyl-labelled isotope. In this way it should be possible to identify some of the unknown metabolites which under these circumstances would be tagged. Experiments would be made on four smokers (2 inhalers, 2 non-inhalers) and four dogs. Data of Fig. 11 and Fig. 12 would be obtained from the experiments on human smokers and the experiments on dogs would provide absorption and elimination data on one and the same animal. No tissue determinations would be done initially. I think this is an interesting project and if properly carried out it might produce some important results.



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27th February, 1964

Re. 25/2/64

PRIVATE AND CONFIDENTIAL

Sir Charles Ellis, F.R.S.,  
British-American Tobacco Co. Ltd.,  
Westminster House,  
7, Millbank,  
London, S.W.1.

Dear Sir Charles,

According to Dr. Geissbühler the statement you  
made at the Brown's Hotel on February 11th, 1964,  
regarding the HIPPO PROJECT was as follows:

"The impetus of HIPPO work has helped the  
tobacco industry to take a positive attitude  
to the beneficial effects of nicotine. We  
have used the general results of this work  
repeatedly in this direction, and as regards  
BAT/Battelle we are entirely satisfied.

We are aware, however, that it is difficult  
to maintain the scientific value of HIPPO  
work against skilled criticism."

With kindest regards

Yours sincerely,  
Walker.

WS/RS

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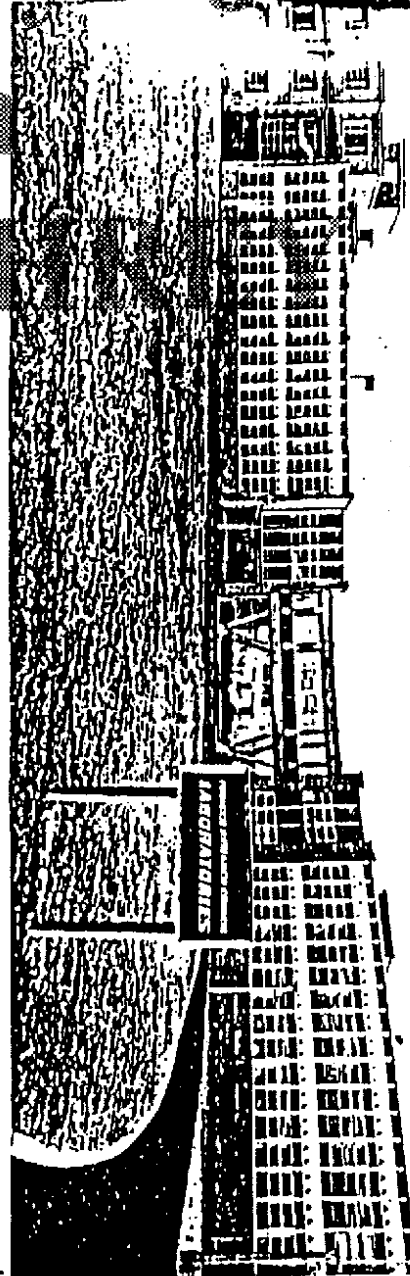
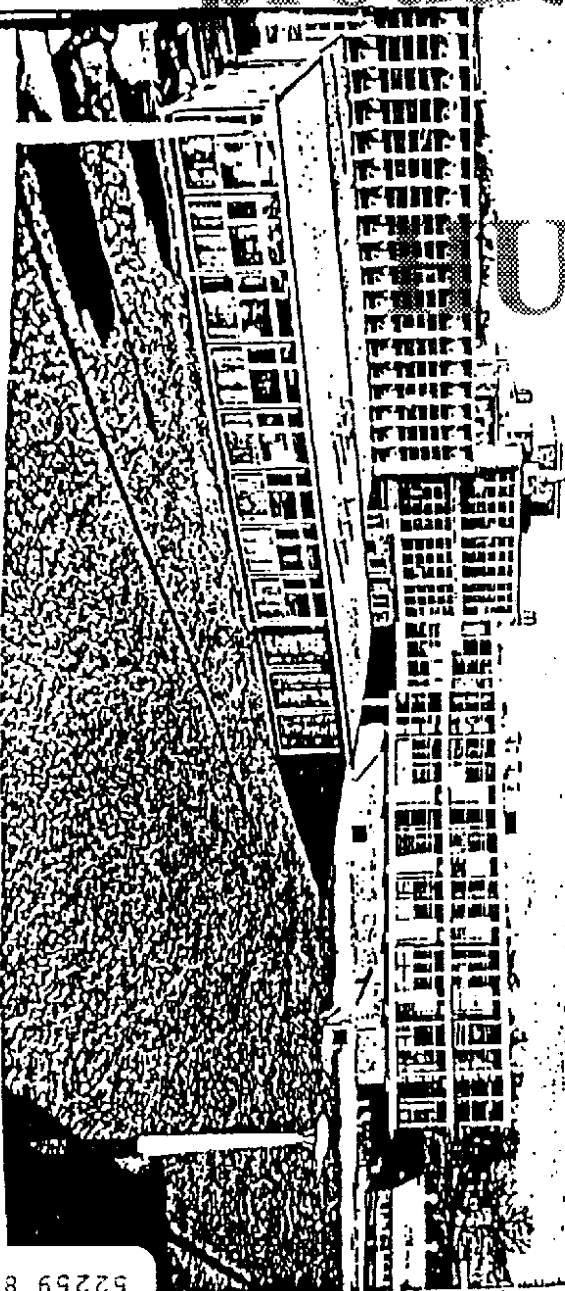
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*The Council's Committees,  
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Professor W. V. Mayneord, FRS

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# Introduction

A *Review of Past and Current Activities* was published by the Tobacco Research Council in January 1963. Although the results of a considerable volume of research on various aspects of smoking and health have been published since then, either by the Council itself or by scientific workers assisted by it, the present *Review* is the first comprehensive account of the Council's activities since 1963. The various projects and experiments to which it refers in broad outline are described in greater detail in the *Summaries of Research Projects and Experiments* (pp. 28-59).

The British tobacco industry has supported research into smoking and health since 1954, and a permanent organisation, then known as the Tobacco Manufacturers' Standing Committee, was set up in 1956. The past few years, however, have seen a very considerable increase in the volume of research and important changes in the manner in which it is conducted; and it was for this reason that the name Tobacco Research Council was adopted in 1963. Every company manufacturing tobacco products in the United Kingdom, whether for the home trade or for export, is directly or indirectly represented on the Council, and on the Committee.

According to its constitution the primary objectives of the Council like those of its predecessor, are "to conduct, promote and co-operate in and keep in touch with research into all questions concerning the relationship between tobacco smoking and health". In its early days the Tobacco Manufacturers' Standing Committee's approach to these objectives was to finance, or assist in financing, relevant projects initiated by other research organisations or individual workers, to furnish them with materials, such as condensate from cigarette smoke, and to provide chemical and statistical information. Since then the Committee, and latterly the Council, has taken an increasing part in initiating new projects and has to a large extent assumed responsibility for carrying them out. The turning-point in this development came in 1961, when it was decided to build laboratories at Harrogate. These came into operation in September 1962, and have since been greatly enlarged.

The Council's staff, at Harrogate and at its headquarters in London, now numbers about 150. Expenditure during each of the past two years, including capital expenditure at Harrogate and grants to independent scientists, has been about £750,000. In addition, a considerable amount of research is carried out in the laboratories of Member Companies.

The manufacturers directly represented on the Council have agreed to pool all information arising from their research in the UK that might be significant for health. Agreements to exchange information have also been concluded during the period between the Council and similar organisations

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in Europe. The Council has long had close working relations with The Council for Tobacco Research—USA.

The Council is indebted to the British Empire Cancer Campaign for Research and to the British Heart Foundation, whose scientific advisory committees give advice to the Council on projects in their respective fields when requested.

On 1st April 1963, Sir Philip Rogers, CBE, was appointed Chairman of the Council in succession to Mr E. J. Partridge. Dr T. D. Day, MA, MD, FRC Path, Director of the Council's Laboratories at Harrogate, is retiring and will be succeeded on 1st March, 1967, by Dr F. Dickens, FRS, MA, DSC, PhD DRC. Dr Day will act as a consultant to the Council.

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# General Survey

Produced by RJRTC

So many statistical associations have been adduced between smoking, particularly cigarette smoking, and various diseases and conditions, that the field of research open to the Tobacco Research Council is a very wide one and there are many possible approaches to particular problems. Finance has never been a limiting factor: but it is essential to select individual projects which are not only of the highest scientific quality in themselves but which as far as possible complement each other to form a coherent and constructive programme.

The Council has always taken the view that its first responsibility must be to provide as much relevant information as possible about the chemical nature of tobacco smoke itself and about its possible effects.

For this reason the largest part of its expenditure and effort in recent years has been devoted to the closely related fields of chemical research and biological testing, both of which are now mainly concentrated at Harrogate. The bio-assay work carried out there is primarily concerned with the possible roles of cigarette smoking in lung cancer. Most of this work is accordingly planned on the working hypothesis that cigarette smoke affects the respiratory epithelium by direct contact. Useful progress has been made in narrowing down the fractions of smoke condensate which are mainly responsible for carcinogenicity as measured by one series of tests, on the skin of mice. Other types of test are in progress or under development. Some of this work is also relevant to bronchitis.

The second main line of research is concerned with people. It comprises a series of studies of widely different kinds to provide information about personal, environmental and other factors in the diseases associated with smoking. These studies cover not only lung cancer but also bronchitis and heart disease and, to a less extent, some other conditions. Information is beginning to emerge about the characteristics of sufferers from these diseases which may ultimately explain why only a minority of smokers and some non-smokers contract any one of them and may make it possible for the most susceptible to be identified.

There is a third group of projects which is on a smaller scale and directed at the motives for smoking. The reason why people smoke is obscure and is likely to be the result of a complex interaction of psychological factors and objective pharmacological effects. The Council is conducting research in both these fields.

The Council also makes a contribution to general medical research not specifically directed towards these various objectives. Finally, it publishes background information, mainly statistical material on smoking and smoking habits, for the benefit of workers in this field.

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There are at present about 20 million cigarette smokers in this country and about one million adults take up or resume smoking each year. The Royal College of Physicians pointed out, in its report on *Smoking and Health* in 1962, that "there can, of course, be no question of prohibiting a habit which most smokers enjoy without injury to their health. . . ."; and two years later the US Surgeon General's Advisory Committee asked "What would satisfy the psychological needs of the 70 million Americans who smoked in 1963 if they were suddenly deprived of tobacco?" but did not offer an answer. It is in the belief that a very large number of people will continue to smoke that the Tobacco Research Council is seeking to provide scientific information which will contribute to a practical solution of the problems involved.

#### BIO-ASSAY AND CHEMICAL RESEARCH

##### TRC Laboratories, Harrogate

Biological and chemical research at Harrogate are closely integrated in the development of animal tests as measures of the carcinogenic and irritant effects of whole smoke, smoke condensate and fractions of smoke condensate. The immediate purpose is to identify any components of cigarette smoke that may be quantitatively important contributors to these effects.

It has been realised from the experience of other research workers in this field that it is necessary to cover a substantial range of biological test systems using different animals, different tissues and different methods of dosing to ensure that the biological activity is measured under a variety of conditions and mechanisms. For this reason a number of biological test systems are being studied and developed at Harrogate. These systems have been selected to test both smoke condensate and whole smoke and are summarised below.

The first group of tests can be described as long term with the possibility of revealing significant changes, and perhaps ultimately the formation of tumours in the exposed tissue. The second group constitutes short term tests and may be of value in the assessment of the irritant effect of cigarette smoke.

##### Long term tests (2-3 years)

1. Application of smoke condensate to mouse skin.
2. Application of smoke condensate to rat lung and rat trachea.
3. Inhalation of whole smoke by laboratory animals.

##### Short term tests

Action of whole smoke on cilia of rabbit trachea (*in vitro*).

At present all these tests with the exception of mouse skin tests are still under development.

The mouse skin test was selected for use at Harrogate for several reasons. At the time that the Harrogate bio-assay work was planned, the opinion of independent advisers was sought on experimental means to test quantitatively the carcinogenicity of cigarette smoke condensate. The Council was advised that pending the development of means to produce bronchial cancer in laboratory animals with cigarette smoke, any animal test in which smoke condensate

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unambiguously gave rise to cancers was worth considering. It was known that dried cigarette smoke condensate that had undergone storage, when applied to mouse skin, could produce epithelial cancers similar in type to the most frequent type of bronchial cancer in man. In addition, there were good prospects of making this test quantitatively reproducible as an aid to achieving the objectives stated in the first paragraph of this section of the report.

The aim of the first test was to determine, so far as experimental limitations allowed, whether the mouse skin carcinogenicity detected by earlier workers was an artifact of processing of smoke following collection or a trivial remnant of a much more powerful effect possessed by fresher condensate. Three materials were examined, stored condensate, 24 hour old condensate, and a neutral fraction derived from condensate. The experiment involved application of the three preparations to the skin of a specific strain of mice over the whole life span of the animals. The experiment was statistically planned and approximately 8,000 mice were used. Great attention was paid to animal husbandry, production of smoke condensate and neutral fraction and the dosing of the animals. Full histopathology and postmortem examinations were carried out on each animal. The data from the experiment were subjected to a full statistical analysis. The results confirmed that cigarette smoke condensate was capable of producing skin tumours in a proportion of mice of the particular strain used in the experiment. The results also suggested firstly that the non-volatile neutral components of cigarette smoke contributed substantially to the mouse-skin carcinogenicity of the condensate; and secondly that the compounds responsible for this effect were stable and not produced as artifacts of processing. The work is now being extended to the study of other tobacco types and products, including cigars. Further details are given in the *Summaries of Research Projects and Experiments*.

The reasons for selecting tests based on the application of smoke condensate to mouse skin have been summarised above. At the same time, it is recognised that these tests have certain limitations. The physical state and chemical composition of smoke condensate differ from those of whole smoke. In particular, no account can be taken of the effects of the more volatile smoke constituents which are lost during the preparation of the condensate. A time interval must elapse between the production and application of the condensate to mouse skin that is much greater than the time taken by smoke to pass from the lips to the lungs of a smoker. Mouse skin tissue differs substantially from human lung tissue. Such differences make it difficult to assess the relevance of these experiments may have to human lung cancer, but they do not affect the value of the experiments in establishing, with considerable accuracy, certain results upon which further research can be based.

In addition to studying the effects of smoke condensate on mouse skin, the effects of applying smoke condensate to the lung tissue of albino rats are being examined, using the methods of Professor J. W. S. Blacklock and Dr L. M. Shabad.

To enable the examination of whole smoke to be undertaken, inhalation techniques using mice have been studied. These experiments involve the regular inhalation of relatively fresh whole smoke by batches of mice using

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equipment based on a design developed by Dr D. W. Henderson, and subsequently modified by Dr R. J. C. Harris of the Imperial Cancer Research Fund. Up to ten smoking sessions per week have been achieved without undue toxicity from nicotine.

Short term tests to measure the ciliastatic effect of whole smoke are also being developed using the rabbit trachea. Part of the human respiratory tract is lined with cilia which beat so as to move bronchial mucus upwards to the throat. The effect of cigarette smoke in arresting the action of the cilia in these tests may throw more light on the action of smoke constituents which are regarded as irritants.

The Chemistry Laboratories at Harrogate play a major role in supplying condensate and fractions of condensate for this bio-assay. Smaller quantities are also supplied to independent scientists. This condensate is prepared under strictly controlled conditions.

The Chemistry Laboratories are also engaged in research on methods to fractionate smoke condensate with the object of demonstrating which chemical constituents of smoke contribute to the biological activity recorded in these tests. This work requires for its development, methods of analysis for certain constituents of smoke, in particular the polycyclic hydrocarbons. Collaborative work in the preparation of standard analytical methods has been undertaken with Member Companies.

At present, the condensates used for skin experiments are not less than 24 hours old. Because the chemical composition of this condensate may be very different from very fresh condensate, a machine has been designed and constructed for the Council by the Battelle Institute, Frankfurt. This machine will deliver applications of condensate to the backs of mice within one minute of the cigarette being smoked.

#### *Bio-assay projects by independent scientists*

The Council has wholly or partly financed or supplied material for certain bio-assay work undertaken by other scientists.

These projects are described in some detail in the *Summaries of Research Projects and Experiments*. Certain of the projects are related to work being carried out in the Harrogate Laboratories.

A series of experiments, supervised by a committee consisting of scientists from the Council and of other scientists co-operating on an individual basis, is concerned with comparing the effects of two methods of curing (flue curing and air curing) tobacco grown from the same type of seed. Mouse skin painting undertaken by Dr F. J. C. Roe is being used to assess the condensate from both lots of tobacco. Inhalation experiments are being carried out by Dr R. J. C. Harris and Dr G. Negroni using mice exposed to whole cigarette smoke, influenza virus and benz(a)pyrene.

Professor L. Lamerton and Dr F. J. C. Roe have recently begun to study the effect of inhalation of tobacco smoke on the pattern of cell proliferation in the bronchial epithelium of small animals using radioactive techniques.

Professor G. A. H. Butler conducted a three-year investigation on the hypothesis that nicotine has the indirect effect of releasing hydrocortisone,

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which in turn reduced the body's defences against cancer; but the results did not support the hypothesis.

#### *Agricultural chemicals*

The Tobacco Advisory Committee, which is concerned only with commercial policy, has sought the advice of the Council in relation to agricultural chemicals applied to growing tobacco. A suggested scheme, involving the biological testing of condensates from cigarettes made from tobacco treated with particular chemicals, has been adopted by the TAC and was officially approved by the Tobacco Research Board of Rhodesia in 1961. Several chemical manufacturers tested their products according to the scheme and two products had completed and passed the tests.

#### PERSONAL, ENVIRONMENTAL AND OTHER FACTORS IN DISEASES ASSOCIATED WITH SMOKING

The main purpose of the research supported or commissioned by the Council in the field of personal, environmental and other factors in diseases associated with smoking has been to obtain more information about the factors, including smoking, which may be either causally or coincidentally associated with these diseases. As many of the studies supported have been statistical in nature, their results do not by themselves enable deductions to be made directly about the nature of the statistical associations between the diseases and the associated factors. The results do, however, contribute valuable information to be taken into account with information from other sources when the nature of the associations is being considered.

In addition to providing more information about the extent of the associations between the factors and diseases examined, the studies commissioned or supported by the Council have helped to throw further light on the characteristics of the individuals who develop each of the diseases concerned. Only a minority of smokers develops any one of the diseases associated with smoking. On this subject, Dr A. Smith wrote in the *Annual Report of the Registrar General for Scotland, 1963*, that "Lung cancer, like many of the epidemic diseases of the present era, derives from environmental factors residing in individual behaviour and is thus not susceptible to public control unless a consensus exists that the environmental influence is undesirable. But whereas it may be virtually impossible to legislate generally against a pleasurable habit carrying no great risk to a majority of the public it might be very much easier to advise against smoking by individuals if those carrying a high risk could be identified. We are still a long way from identification of such individuals in the case of lung cancer but in coronary heart disease this is already a practical possibility". It is hoped, however, that some of the research projects supported by the Council may have brought closer an accurate description of the characteristics of individuals who fall into the highest lung cancer risk groups.

During the period under review the Council has financed, wholly or partly, twenty-five separate external projects concerned with various factors in diseases or conditions which have been statistically associated with smoking.

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The cardio-respiratory disease research project of the Department of Medical Statistics and Epidemiology of the London School of Hygiene and Tropical Medicine is the largest in this field and the largest external research project supported by the Council. Thirteen other projects are concerned with lung cancer and/or bronchitis and are mainly epidemiological in character. Ten are concerned with heart disease and are supported by the Council for the most part on the recommendation of the British Heart Foundation. The remaining one is concerned with the effects of smoking and other factors in pregnancy.

*Cardio-respiratory disease research project*

The Council agreed to contribute up to £500,000 over the ten years from 1st August 1963 to the London School of Hygiene and Tropical Medicine, so as to enable the Department of Medical Statistics and Epidemiology at the School to be expanded in order to carry out a cardio-respiratory research project proposed by Professor D. D. Reid. The main aim of the project was to obtain more information about the personal and environmental factors affecting the onset and development of the commoner chest and heart diseases. One of the objectives was to help doctors to identify patients unduly susceptible to these diseases so that timely and appropriate advice could be given. The project consists of a series of interlocking studies, each designed to illuminate some aspects of the subject, and forms an integral part of the work in the cardio-respiratory field of the Department of Medical Statistics and Epidemiology, which is also supported by the Public Health Service of the United States.

The several studies of the Department differ in scale, duration and technique and are designed not only to provide information on specific points but to contribute to the broad objectives. The most important so far has been the British portion of an international study covering Britain, Norway and the United States and stemming from the observation that among men in middle life the American death rate from coronary heart disease is 50 per cent greater than in this country but from lung cancer and bronchitis only half as high. Preliminary inquiries have been followed by a detailed comparison of the medical histories of British and Norwegian emigrants to America, of their relatives who remained at home and of a random sample of British residents. These have shown, for example, that the Norwegian-born American suffers less from the diseases in question than the British-born, whose chances of dying of chronic bronchitis are reduced by emigration but who apparently carries with him a high though somewhat reduced risk of lung cancer compared with those who remain in Britain.

The suggestion that the seeds of respiratory disease are sown in childhood has prompted investigation among school-children in Sheffield and rural Glamorgan, which is being followed by a much larger survey.

As a means of distinguishing genetic from environmental factors, studies have been made of the medical histories of identical and non-identical twins in Denmark and of families in this country. Past medical records of Post Office workers who have contracted lung cancer have been compared with those of men doing the same job who have not, and Royal Air Force records

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are being studied with reference to coronary heart disease; routine sickness records in such organisations may provide an initial screen to indicate men most likely to contract these diseases. Radiographic and electrocardiographic records and measurements of lung function, blood pressure and blood sugar have been taken for various groups of men, whose future medical experience will be followed up.

The effects of smoking and other factors at times of special physical stress are being examined in studies of sufferers from heart infarction and others.

Special techniques for the handling of this great mass of data by computer and for its detailed statistical analysis are being developed.

#### *Lung cancer and bronchitis*

A study of deaths from lung cancer and bronchitis in Northern Ireland in 1960-2 was carried out by Dr G. Dean and AGB Research Ltd. AGB Research Ltd earlier carried out a similar survey in areas of North East England for the years 1952-62. The results of both studies have been published. They showed statistical associations between each disease and both smoking and urban residence, but the strength of these associations varied. In both areas, lung cancer mortality was more strongly associated with smoking habits than with urban residence. In the areas of North East England covered by the inquiry, bronchitis mortality was more closely associated with urban residence than with smoking; in Northern Ireland, bronchitis mortality was associated about equally with these two factors.

The data from these two studies was used in a further study in which mortality models were constructed in an attempt to define more precisely the relative importance of the different characteristics that can now be used to classify men according to their risk of dying from lung cancer or bronchitis. In *TRC Research Paper No. 7*, mortality from lung cancer and bronchitis in 219 districts of England and Wales was examined in relation to smoke and sulphur dioxide concentration, population density and social index. One important finding was an association between mortality from these causes and population density.

Dr G. Dean investigated the country of birth and smoking habits of people who had died of lung cancer in the Channel Islands from 1952 to 1961. The results, which have been published, showed that mortality was higher in Jersey than in the other islands, for reasons which are unexplained. During the period under review Dr Dean also carried out a follow-up study which confirmed his earlier work on lung cancer in rural South Africa, and a comparative study of all causes of death among locally-born South Africans, immigrants to South Africa and residents of the United Kingdom: the results of both have been published.

The possible relationship between bronchitis and lung cancer is being studied by Dr W. E. H. Field and others, using indices of mucus gland hypertrophy, in hospital patients in the Gloucester area.

Professor C. V. Harrison, Dr S. W. A. Kuper, and Dr P. Stradling of the Royal Postgraduate Medical School, London, are investigating the possibility of detecting pre-malignant changes in the respiratory tract by means of exfoliative cytology techniques applied to sputum. Dr Lynne Reid and Dr E. E. Keal

of the Institute of Diseases of the Chest are measuring mucus production in bronchitics and others, relating its neuraminic acid content to certain factors.

Other investigations are described in the *Summaries of Research Projects and Experiments*.

#### *Cardiovascular disease*

Some research of an epidemiological nature in the field of cardiovascular disease forms part of the interlinked studies of the cardio-respiratory disease research project mentioned above. In addition the Council has supported investigations at a fundamental research level, mainly on the recommendation of the British Heart Foundation, into the causes of those forms of heart and arterial disease which have been associated with smoking. Only one of these investigations is concerned specifically with the effects of smoking, and another took account of smoking among other factors.

Six projects are concerned with the development of atherosclerosis, a disease of the arteries which frequently underlies the process of coronary thrombosis, two are concerned with peripheral vascular disease and another with diseases of the small blood vessels as exemplified by the retina of the eye. All of these are in an early stage and are described in more detail in the *Summaries of Research Projects and Experiments*.

The Council has contributed throughout the period under review to the work of the Medical Research Council's Cardiovascular Research Group under Dr. J. P. Shillingford at the Royal Postgraduate Medical School, London. The Group has studied the effect of smoking on blood flow in normal subjects and in patients with coronary heart disease or valvular disease. The results of an early series of experiments indicated that cigarette smoking, especially with inhaling, could be harmful in cases of coronary thrombosis or of incompetence of the aortic or mitral valves. The effects of nicotine on peripheral blood flow are being studied.

#### *Birth-weight and perinatal mortality*

The Council has contributed to the cost of an analysis, not yet quite completed, of data collected by the National Birthday Trust Fund during its investigation of birth data in March-May 1953. This covers various factors, including smoking, that may be related to birth weights, still births and deaths of infants up to one month of age.

#### PSYCHOLOGICAL ASPECTS OF SMOKING

"The benefits of smoking", the Royal College of Physicians stated in their report on *Smoking and Health* (1962), "appear to be psychological and social and are hard to express in quantitative terms." On the other hand, according to the report on *Smoking and Health* of the Advisory Committee of the US Surgeon General (1964), "the habitual use of tobacco is related primarily to psychological and social drives, reinforced and perpetuated by the pharmacological actions of nicotine on the central nervous system, the latter being

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interpreted subjectively as stimulant or tranquillising dependent upon the individual response".

The Council has supported research into both psychological and pharmacological aspects of smoking. Any valid analysis of the motives for smoking must be supported by or at least be consistent with both the psychological and pharmacological evidence.

The Council agreed in 1964 to participate in a study by Dr F. E. Emery of the Tavistock Institute of Human Relations, carried out in co-operation with Public Attitude Surveys Ltd, involving 2,500 interviews. Preliminary results implied that the main motives for smoking were the needs to concentrate, to offset boredom and to relieve tension, in that order.

Since 1961 Professor H. J. Eysenck of the Institute of Psychiatry has been conducting a series of experiments on the behavioural effects of nicotine, as a follow-up to earlier studies on stimulant drugs. These experiments involved both rats and human beings and produced variable results. Professor Eysenck has suggested that nicotine may aid "consolidation" during periods of rest in mental tasks, in the sense that associations become fixed and learning and memory are enhanced. He also concluded that nicotine might have a stimulating effect that was a prime factor in the continuation of the smoking habit once it had developed.

#### PHARMACOLOGICAL ASPECTS OF SMOKING

The purpose of the Council in carrying out and supporting pharmacological research is to provide some reliable information on those pharmacological effects of smoking that may throw some light on the reasons why people smoke. In particular it is the aim of much of this research to see what experimental facts can be adduced to verify the widely held subjective impression of smokers that smoking has both stimulating and tranquillising effects. There are reasonable grounds for believing that nicotine is by far the most important constituent of tobacco smoke from this point of view. Since the amounts of nicotine absorbed in smoking are relatively small, work is being directed to the effects of administering small doses of the alkaloid. A further aim is to improve knowledge of the pathways by which the central and peripheral effects of small doses of nicotine are mediated.

##### *Pharmacological research: TRC Laboratories, Harrogate*

Work began here on a small scale in 1963. On the recommendation of Professor J. H. D. work has included studies of the effects of nicotine on the central nervous system, of nicotine and of tobacco smoke on blood pressure and the release of catecholamines, and of nicotine on blood glucose and free fatty acid levels. Effects of nicotine on the behaviour of rats and mice in a series of experiments involving the measurement of motor activity and operant conditioning procedures have also been investigated. First results support the view that one effect in the brain of the administration of small doses of nicotine is to release acetylcholine which suggests a mechanism whereby smoking might well help in maintaining normal brain function. The effects of small doses of nicotine on the behaviour of rats which have

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been trained to work for rewards are consistent with there being a pharmacological basis for the experience of smokers that smoking helps them to concentrate more effectively on a task that demands close attention over a period of time.

The Pharmacological Laboratories have recently been expanded in order to develop further these promising lines of research.

*Pharmacological research by independent scientists*

Dr M. S. G. Clark and Dr S. K. Vanov of the London School of Pharmacy have studied some central and peripheral effects of nicotine and related alkaloids. Dr Clark has suggested that the level of motoneurone activity and muscle tone are liable to increase in a stressful situation. Nicotine absorbed during smoking may reduce motoneurone activity by stimulating an inhibitory system (the Renshaw cell) in the spinal cord, or by an effect at higher levels, possibly by acting on structures associated with the reticular formation.

Dr R. B. Barlow of Edinburgh University is at present studying the relationship between chemical structures and nicotine-like activity; Professor A. H. of Chelsea College of Science and Technology has started a study of nicotine absorption and metabolism in man; and Dr R. E. Marley of the Institute of Psychiatry, London, is investigating the effects of nicotine-like and related compounds on the central nervous system of chickens.

GENERAL MEDICAL RESEARCH RELEVANT TO  
SMOKING AND HEALTH

*Immunology and cancer*

The Council contributed support for five years, on the personal recommendation of Professor Sir Alexander Haddow of the Institute of Cancer Research, to research carried out by Dr J. F. A. P. Miller into the functions of the thymus and particularly into its contribution to natural resistance to tumours. Dr Miller has described his results in a number of papers.

The Council also supported research by Professor Sir Alexander Haddow himself into the regression of chemically-induced tumours and into immunological mechanisms in the rat.

BACKGROUND INFORMATION RELATING TO SMOKING

- As part of its work in making information available to scientific workers and others, the Council has published further reports in its series of *Research Papers* in addition to those mentioned elsewhere. *Tobacco Consumption in Various Countries*, covering twenty-eight countries, was published in 1963. *Cigarette Smoking Characteristics in the UK, South Africa and Australia*, also published in 1963, was the report of an inquiry into possible differences which might have invalidated the comparisons of lung cancer mortality made by Dr G. Dean, but the differences were found to be minor. In 1966 a supplement was published to a report on *The Reliability of Statements about*

*Smoking Habits*, published in 1958. A second supplement to *The Constituents of Tobacco Smoke* was published in 1963, and a third supplement is being prepared. A fourth edition of *Statistics of Smoking in the United Kingdom* has been published.

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*Summaries of research  
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## Part I. TOBACCO SMOKE: BIO-ASSAY

### *Section 1. TRC Laboratories, Harrogate*

The aims of the bio-assay work at the TRC laboratories at Harrogate have been outlined in the *General Survey*.

#### 1. Mouse skin tests for tumorigenicity

When the bio-assay tests were started at the Council's laboratories it was known that dried and aged cigarette smoke condensate (commonly but erroneously called "tar") was capable of causing epithelial tumours in certain strains of mice. This had led to the belief that the agents in cigarette smoke responsible for causing mouse skin tumours were stable non-volatile compounds. Little account had however been taken of the possibilities

- (a) that moderately volatile cigarette smoke constituents might contribute appreciably to mouse skin tumorigenicity of smoke condensate, and
- (b) that the drying and ageing processes involved in preparing and storing condensates might alter their tumorigenicity either through the destruction of unstable carcinogens or by the production of carcinogenic artefacts.

Further, it was not known with any degree of precision what proportion of the mouse skin tumorigenicity of cigarette smoke condensate was due to neutral components of the condensate and what proportion to the acidic or basic components, which some workers had suggested might have important tumour promoting effects.

The aim of the first experiment at the Harrogate laboratories was to investigate these problems using the best experimental techniques available at the time, on a scale sufficient to give the numerical results a precision hitherto unattained in this field.

The mouse skin tumorigenicity of condensate from a composite plain cigarette blend, chosen to be typical of UK manufacture, was tested using three types of cigarette smoke condensate:

- (a) *24-hour condensate*. Smoke condensate collected in a cold trap was dissolved in acetone and concentrated at 40°C under reduced pressure without being taken to dryness. It was applied within 24 hours of smoking the cigarettes.
- (b) *Stored condensate*. Smoke condensate solution was evaporated to dryness on a boiling water bath and stored at -29°C for several weeks before being re-dissolved in aqueous acetone for skin application.
- (c) *Neutral fraction*. Acids and bases were removed from smoke condensate and the neutral residue dissolved in aqueous acetone for skin application.

A total of 3,000 mice, used in groups of 1,000 at quarterly intervals, was employed to compare the skin tumorigenicities of these three materials, 2,000 of the animals being divided between untreated controls and controls treated with aqueous acetone alone. Special attention was paid to animal husbandry, firstly in order to enable the stock to be kept as disease-free as possible and secondly to minimise the introduction of systematic differences in the handling and treatment of the animals which might reduce the precision of the results. The mice were females of a specific pathogen-free albino strain which was found to combine tolerance for nicotine with adequate response to skin application of cigarette smoke condensate. The skin tumour response to benz(a)pyrene and dibenz(a,h)anthracene was used to check the uniformity of successive deliveries of mice.

Animals treated with condensates were divided equally between 3 dose levels with dose ratio 1:2:4, equivalent to 25 mg, 50 mg, and 100 mg applications of dried condensate. Pilot tests had suggested that 100 mg was about the highest dose that could be adequately tolerated over a long period of time. Condensates were applied to each animal three times a week. Tumorigenic response was judged by the count at regular intervals of the presence in the treated area of the skin of papilloma, muscle-infiltrating carcinoma and carcinoma not infiltrating muscle as defined by strict histological criteria so far as possible independent of subjective judgement. The results of this experiment (Day, 1967) have confirmed that cigarette smoke condensate is capable of producing all three types of tumours in varying proportions of the mice of the particular strain used and that there is a dose-response relationship. The relative tumorigenicities of the three condensates were calculated in two different ways—

- (a) From age-standardised rates for tumour-bearing animals after 128 weeks treatment, and
- (b) From the distribution over time of the "tumorigenic force", defined as the number of new tumour-bearing animals found in a short period divided by the number of tumourless animals alive in the group at the start of the period.

These procedures were adopted, rather than the proportion of tumour-bearing animals used by most other workers, because there were marked differences in life expectation between treatments. The second method also confirmed that the tumour response curves plotted against time fitted the same theoretical model for all three condensates and dose levels from which it was a reasonable presumption that the condensates acted in broadly the same way on the test animal. This enabled a valid comparison of tumorigenicity to be made. Analysis showed that relative tumorigenicities calculated for each of the three tumour types by the two alternative procedures agreed within the confidence limits of the numerical results. The results suggested that non-volatile neutral components account for something more than 50 per cent of the tumorigenicity of 24-hour condensate and 80 per cent of that of stored condensate as defined by the experimental procedures. It follows that the compounds responsible for this effect are stable after collection for several weeks and are not affected by moderate heat treatment and

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chemical manipulation; it is unlikely that they are produced as artifacts in the process of making stored condensate. The results suggest that 24-hour condensate is more tumorigenic than stored condensate. While neither material contained what are usually termed "volatile" smoke constituents there must be substances in acetone solutions of 24-hour condensate which are lost or modified when evaporation is carried out to dryness; chemical analyses of these condensates are however not informative because at this stage it is not known to which of the large number of constituents attention should be directed. The study provides no evidence to decide whether any part of the activity of 24-hour condensate is due to its processing or short storage.

In practical terms an important feature of these results seems to be that they show that in relation to mouse skin there are stable non-volatile neutral carcinogens in cigarette smoke condensate which are worth serious attention and which in particular merit investigation by detailed fractionation.

Further experiments are now in hand in an attempt to discover if the mouse skin tumorigenicity of cigarette smoke condensate neutral fraction is associated with any particular class of compounds in this fraction. The first indications from these tests, which are not yet complete, are consistent with much of the tumorigenicity of cigarette smoke condensate neutral fraction being due to polycyclic aromatic hydrocarbons or their near relatives.

Further work is also in progress in an attempt

- (a) to shorten the time between smoke condensate collection and skin application from 24 hours, which was the shortest interval attainable with the smoke collection apparatus available four years ago, to an interval of the order of one minute, and
- (b) to obviate the need for a concentration stage in preparing solutions of freshly prepared condensate for skin application and thereby to retain in these solutions a greater proportion than has hitherto been possible of smoke constituents of moderate volatility.

As a practical step towards the possible utilisation of the information obtained in the first experiment, tests are also in hand at the Harrogate laboratories with experimental cigarettes in which the yield of non-volatile smoke condensate has been reduced and in which the ratio of the yield of non-volatile smoke condensate to the yield of nicotine has been reduced.

## 2. Other experimental techniques

Exploratory work has been undertaken to investigate the effects on animals of cigarette smoke condensate applied by injection and intubation techniques and of inhaled smoke respectively.

A procedure devised and used by Professor J. W. S. Blacklock (Blacklock, 1961; Blacklock and Burgan, 1962) for applying cigarette smoke condensate to the rat lung is being developed. Intratracheal application of smoke condensate to rats by a technique similar to that developed by Dr L. M. Shabad of Moscow (Shabad 1962) is also being used.

To enable the effects of smoke in aerosol form, as distinct from condensed

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smoke, to be examined, inhalation techniques are being studied. Mice are exposed to cigarette smoke in an apparatus developed by Dr D. W. Henderson, and subsequently modified by Dr R. J. C. Harris of the Imperial Cancer Research Fund. A standard puff of 25 ml. cigarette smoke is diluted and expelled into a chamber so designed that twenty mice are exposed simultaneously to the smoke. The chamber is tubular and each fresh puff of diluted smoke displaces the previous one. The first groups of mice were given three exposures per week and, with a view to investigating whether it was possible for mice to tolerate higher dose levels, further groups were given five or ten exposures per week. The experiments are still in progress.

The smoke inhalation experiments provide the possibility of observing changes which may lead to cancer. The aim in these experiments is to have the smoking conditions as near to those of average human smoking as possible, but there are difficulties due to the relatively short life span of experimental animals and the differences between the types of cells in the rodent and human lungs.

Tests of carcinogenicity using mice or rats normally last for the whole life span of the animal and require two or three years, excluding the time required to analyse the results. The value of shorter term tests is therefore being investigated. In one test, cigarette smoke condensate as a whole or in fractions, or known carcinogens, are applied to mouse skin and the thickening of the epidermal layer of the skin that occurs after a few days after the first dose is measured. In another test, tissue culture techniques are being used. Neither technique has yet been fully developed.

### 3. Tests of ciliastasis

As part of the investigation of the effects of cigarette smoke on respiratory tissue, a test for assessing the ciliastatic effect of cigarette smoke by using rabbit trachea is being carried out. The ciliastatic effect of cigarette smoke in this test is reduced if either the particulate matter or certain vapour phase constituents of the smoke are reduced. It is intended to examine the smoke from cigarettes with different types of filters.

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## Section 2. Bio-assay Projects by Independent Scientists

### 1. Comparison of flue-cured and air-cured tobaccos

As reported in the 1963 *Review of Past and Current Activities*, the Council is financing a study of the properties of smoke from cigarettes made from flue-cured and bulk-fermented air-cured tobacco in order to compare the effects of the two types of curing. Tobacco plants were specially grown from the same seed under controlled conditions in Mexico. Part of the resulting leaf was treated by flue-curing and part by air-curing and bulk fermentation, and cigarettes were manufactured to standard specifications from each batch. Some of the cigarettes were smoked and condensate produced at TRC Laboratories at Harrogate. The quantities of certain constituents of the tobacco and the smoke were estimated by the Research Departments of Member Companies.

Mouse skin painting experiments with condensates from these cigarettes were started towards the end of 1963 under the direction of Dr F. J. C. Roe of the Institute of Cancer Research. The experiments have been completed but the results await detailed statistical analysis.

In addition, pilot inhalation experiments are being carried out by Dr R. J. C. Harris and Dr G. Megroni of the Imperial Cancer Research Fund, using cigarette smoke on C 57 black mice, alone or in conjunction with influenza virus. Mice have also been exposed to influenza virus alone or to benz(a)pyrene alone or following influenza virus infection. Some tumours have been produced but the experiment is still in progress and it is not possible to draw final conclusions.

### 2. Biological tests of carcinogenic activity

(Professor E. Dickens, Dr H. E. H. Jones, and Mr H. B. Waynforth, Courtauld Institute of Biochemistry, Middlesex Hospital Medical School)

A technique for administering substances directly by intubation into the lungs of rats has been used to investigate the possible induction of lung cancer in the animals. By means of this technique 40  $\mu$ l of material was placed at the lower end of the trachea, and from there it entered the lungs as the animals breathed. By fluorescence microscopy it has been shown that benz(a)pyrene placed in the trachea all passed into the lungs within a few hours, while none appeared to be regurgitated into the mouth. Tetracycline was given in the drinking water to prevent intercurrent respiratory infection.

Tobacco smoke condensate was instilled once weekly and three times weekly intratracheally under anaesthetic into separate groups of rats for one year. A control group was anaesthetised only. The lungs of these animals were examined histologically when they died or in survivors at the end of a further year. Inflammatory but no neoplastic changes were observed. Administration of the neutral fraction of tobacco condensates in this way, even in very large total amounts (up to 150 mg.), during the course of one year did not give rise to lung tumours in rats during a further year. This negative result contrasts with positive findings in other types of tests.

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Using the same technique, substances which had shown definite carcinogenic activity when injected subcutaneously into rats have been tested for their ability to induce tumours of the rat lung. Beta-propiolactone, penicillic acid, aflatoxins B and G, benz(a)pyrene, and methylprotoanemonin in arachis oil have been supplied by intubation. Rats were examined when they died, and all surviving 100 weeks after the first administration were killed.

All rats given penicillic acid, methylprotoanemonin, benz(a)pyrene, or arachis oil alone, survived for as long as 80 to 100 weeks without any malignant change being observed in the lungs or elsewhere. The result with benz(a)pyrene was considered surprising since each rat received 18 mg. of this potent carcinogen, but it is in accord with the observations of other workers who found that rat lung tissue was highly resistant to polycyclic aromatic carcinogens.

A lung tumour was seen in a rat in the 72nd week after administrations of beta-propiolactone were started. The tumour affected only one lobe of the lung and was described as a keratinising squamous carcinoma of the bronchiolar epithelium. It was of special interest that this lactone produced a carcinoma, whereas benz(a)pyrene in the same dose was inert.

None of the rats given aflatoxin by this route showed malignant growths of the lungs, though this potent carcinogen induced squamous carcinomata of the trachea as well as tumours of the liver, pylorus and kidney.

The results of experiments in rats and mice injected subcutaneously with substances related to the lactones have been published (Dickens and Jones 1965).

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#### 5. Nicotine, corticosteroids and tumours (Professor G. A. H. Buttle, School of Pharmacy, London)

Professor Buttle has examined several aspects of the problem of corticosteroid release by nicotine, the effects of corticosteroids on tumour growth, and the ability of transplanted tumours to establish their growth within the host animal.

In studying the effects of smoking by humans on hydrocortisone levels in blood, Professor Buttle found that in four individuals who smoked up to six cigarettes in quick succession, the diurnal variation of hydrocortisone levels obscured any possible effect due to smoking except in the case of one non-smoker.

When high doses of nicotine were injected into rats it could be shown that there was an increase in the blood corticosterone level. The increase however was small when compared with that brought about by adrenocorticotropic hormone.

In experiments where human tumour material was inoculated into weanling rats, nicotine tartrate given twice daily for seven days from the time of tumour inoculation resulted in failure of the tumours to grow in

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these animals. When a similar experiment was carried out in which the same doses of nicotine were reinforced with one unit of long-acting adrenocorticotrophic hormone daily for seven days, the tumours also failed to grow. Similarly, one unit of adrenocorticotrophic hormone without nicotine did not result in growth, but where ten units daily were administered, there was good growth of the transplanted tumour. It was concluded that nicotine alone did not stimulate the pituitary sufficiently to liberate the quantity of adrenocorticotrophic hormone equivalent to ten units of the long-acting preparation daily. Nor did it appear that nicotine potentiated the action of a small dose of adrenocorticotrophic hormone to any great extent.

When long-acting adrenocorticotrophic hormone was given to rats after the implantation of pellets of benz(a)pyrene, tumours did not appear to develop in any different way from those in the control animals which did not receive the hormone. There was perhaps some slight indication of increased tumour growth with large doses of adrenocorticotrophic hormone, but after two weeks this hormone produced toxic effects, and these obscured the interpretation of the results.

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#### 4. Comparison of cigar and cigarette smoke

(Professor R. D. Passey, Institute of Cancer Research, London)

Professor Passey asked the Council for a supply of cigarettes manufactured wholly from cigar tobacco so that the effects on animals of smoke from cigar tobacco could be compared with those of smoke from cigarettes made from standard flue-cured tobacco.

At first, attempts were made to observe the effects of both types of smoke when applied to the rabbit eye. While, in general, the cigar smoke appeared the less irritating, it was not possible to detect any marked difference when Professor Passey applied both types of smoke in similar manner to his own eyes.

When rats were exposed in an enclosed cabinet to the two types of smoke, marked differences emerged. The flue-cured tobacco smoke caused more deaths and induced more severe changes in the respiratory system (ranging from loss of cilia to bronchopneumonia) than the cigar tobacco smoke which was well tolerated and induced nothing comparable in severity. The lungs of rats exposed to the smoke of cigar tobacco cigarettes for a prolonged period were found at post mortem to differ only slightly from those of a control group. Further experiments have shown that cigarettes made from burley tobacco give results comparable to those made from cigar tobacco.

When the backs of mice were painted with condensate from the smoke of cigarettes made from flue-cured and cigar tobacco respectively, the latter gave rise to papillomata in 30 per cent of the survivors in contrast to no tumours with the flue-cured condensates.

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Professor Passey has suggested that these results are related to the high plant sugar content of flue-cured tobacco in contrast to the low content of the cigar and burley tobaccos.

#### References

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#### 5. Carcinogenesis in new-born mice (Mrs A. Flaks, University of Leeds)

A series of experiments was undertaken in view of the report (O'Gara *et al.*, 1962) that it was possible to shorten the time between the administration of a carcinogen and the appearance of subsequent tumours by using new-born mice. It was considered that this should be a more powerful test than skin painting. The materials used were 9, 10-dimethyl-1, 2-benzanthracene, denitrinised cigarette smoke condensate, and the neutral fraction of cigarette smoke condensate. The results of the tests did not fulfil expectations.

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#### 6. Stimulation of hydroxylation in liver (Dr D. V. Parke, St Mary's Hospital Medical School)

It has been suggested that the carcinogenicity of benz(a)pyrene might be due to its activation of a liver enzyme system which might be a potential producer of carcinogens from ingested foreign chemicals. A small study was therefore started to throw light upon this hypothesis, and a system was used in which the effects of pre-treatment of animals with benz(a)pyrene and phenobarbitone upon the *in vivo* hydroxylation of anisole and fluorobenzene were studied.

Increased toxicity and potential carcinogenicity have been associated with the ortho-hydroxylation of aromatic compounds, especially amines. The enzyme systems responsible for the hydroxylation of aromatic compounds are located in the microsomal fraction of the liver and have been shown to be stimulated by pre-treatment with a variety of chemicals including chlorinated hydrocarbon pesticides, barbiturates and benz(a)pyrene.

The experiments showed, however, that pre-treatment of animals with benz(a)pyrene or phenobarbitone before the administration of fluorobenzene or anisole did not result in an exclusive increase in the metabolism to the corresponding *ortho*-phenols.

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### 7. Respiratory cell proliferation

(Professor L. Lamerton and Dr F. J. C. Roe, Institute of Cancer Research, Sutton)

The aim of these experiments, which have been recently initiated, is to study the effect of inhalation of tobacco smoke on the pattern of the cell proliferation in the bronchial epithelium of small laboratory animals. By applying radio-active techniques to the study of cell population dynamics in the mammalian respiratory tract it is hoped that information will be obtained on cell proliferation parameters such as the mean cell generation time, the proportion of cells which are involved in division and the residence time of cells. It is known from work already carried out that, under the stress of continuous irradiation, new steady states of cell population pattern can be set up in such tissues as gut and bone marrow. It is hoped to learn, among other results, whether a new steady state of cell proliferation is set up under the effects of smoking or whether there is a progressive change in some or all of the parameters indicated above.

### 8. Asbestos, lung cancer and mesothelioma

(Dr F. J. C. Roe and Dr J. S. Harington, Institute of Cancer Research, London)

A number of experiments, supported to a small extent by the Council, have been undertaken to compare the carcinogenicity of crude commercial asbestos with that of pure fibres after the removal of "natural" and "contaminating" oils, including jute oil, which contain a number of polycyclic hydrocarbons such as benz(a)pyrene. Groups of rats and mice were injected subcutaneously respectively with crude amosite, crude crocidolite, cyclohexane-extracted amosite, cyclohexane-extracted crocidolite, or crude chrysotile. Sarcomas at the injection site and mesotheliomas developed in some animals of all groups, and the carcinogenic response to extracted fibres was no less than to the corresponding crude material. Control animals injected with the suspending medium, saline, developed no tumours of either type.

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## Part II. TOBACCO SMOKE: CHEMICAL RESEARCH

### *Section 1. Chemistry Department, TRC Laboratories, Harrogate*

The main tasks of the Chemistry Department are to identify and, where appropriate, to estimate constituents of tobacco smoke relevant to smoking and health problems and to assist the scientific staff of Member Companies in discovering practical means of eliminating or reducing any constituents which may appear hazardous to health. The Chemistry Department is also responsible for the development and supervision of the mass production of smoke condensate and of condensate fractions for biological testing at the Harrogate laboratories and elsewhere.

As indicated in the section in this report on bio-assay, it has been shown that the carcinogenic response of mouse skin to tobacco smoke condensate which has been subjected to heat treatment and subsequent prolonged storage at low temperature is about 60 per cent of the response to material painted only 24 hours after production. Research has therefore been directed at the isolation and identification of the stable carcinogens. Some emphasis has been placed on the aromatic polycyclic hydrocarbons and the aromatic heterocyclic constituents of tobacco smoke condensate, since the few compounds known to have a carcinogenic effect on mouse skin which have so far been isolated from tobacco smoke have largely fallen into these two chemical groups. There is little doubt that the spectrum of smoke constituents falling into these chemical groups, albeit in minute concentration individually, is very wide and there is evidence of the presence of a wide range of methylated polycyclic aromatic hydrocarbons, some of which are known from animal tests to be more carcinogenic than the unmethylated compounds. The amounts of known skin carcinogens in tobacco smoke, however, have been regarded as insufficient to explain the level of carcinogenic effect of stored smoke condensate on the back of a mouse.

The main line of chemical research has been to devise fractionation schemes for whole smoke condensate, firstly to concentrate the polycyclic aromatic hydrocarbon constituents into a single fraction, and secondly to separate these compounds from other non-polycyclic constituents in order to determine whether the stable mouse carcinogens in smoke condensate are members of the former group.

In early work on this problem, the ether-soluble neutral constituents of smoke were obtained by extracting basic, acidic and phenolic materials by washing ether solutions of condensate with aqueous acid and alkali. This neutral material, which accounted for about 33 per cent of whole smoke condensate, was further fractionated by adsorption chromatography on

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alumina columns into a hydrocarbon fraction representing 8 per cent of whole smoke condensate and containing aliphatic hydrocarbons, other long-chain aliphatic compounds and the polycyclic aromatic hydrocarbons, and a non-hydrocarbon fraction which made up 22 per cent of whole smoke condensate.

The results of testing neutral fraction indicated that a slightly lower response was obtained than with the stored smoke condensate. Because of the nature of the fractionation procedure, it was not possible to recover all the extracted material and recombine all the various fractions and hence to test whether this lowering of response was due to mechanical losses, distribution of primary carcinogenic material into other fractions or the removal of promoting agents. Further, since this particular method of fractionation only took into consideration ether soluble material and the bases and acids were isolated as free compounds, little useful information would arise from testing the fractions other than neutral fraction.

In an attempt to overcome the difficulties presented by the procedures in the preparation of neutral fraction, alternative methods have been studied in which similar material could be obtained without the use of strong acids and alkalis and which would ensure as far as practicable the complete recovery of all smoke materials. The latter requirement of the fractionation processes is essential if a complete picture of the carcinogenic response of whole smoke condensate is to be obtained. Tumour yields on mouse skin are possibly determined not only by the amounts of primary carcinogens, promoting agents, co-carcinogens or inhibitors present in the test material, but also by the proportion of the skin surface shielded by the inactive constituents. For a fuller interpretation of the results, therefore, not only should all fractions be tested individually but also recombinations of the fractions.

A number of procedures have been studied for the separation of whole smoke condensate into fractions in which the polycyclic aromatic hydrocarbons are concentrated into a single fraction. These studies have included the water-soluble and water-insoluble constituents of smoke condensate, the distribution of smoke materials between pairs of solvents—cyclohexane and aqueous methanol, cyclohexane and dimethyl sulphoxide, iso-octane and polyglycols, the reduct formation of long chain aliphatic constituents with urea and thiourea, and the adsorption of smoke materials by silica gel and alumina.

By combining a number of these procedures in suitable sequence a fractionation scheme has been evolved which separates whole smoke condensate into seven fractions and which results in the substantial concentration of the polycyclic hydrocarbon constituents into a single fraction representing 0.8 per cent by weight of the original material. This fractionation scheme is summarised in diagram 1.

These studies have involved the development of gas chromatographic techniques using an electron capture detector for the qualitative monitoring of the polycyclic constituents passing into different fractions. Quantitative assessment of the efficiency of the concentration of these compounds has been partially achieved by the tracing of a number of selected polycyclic aromatic hydrocarbons labelled with radioactive carbon or radioactive

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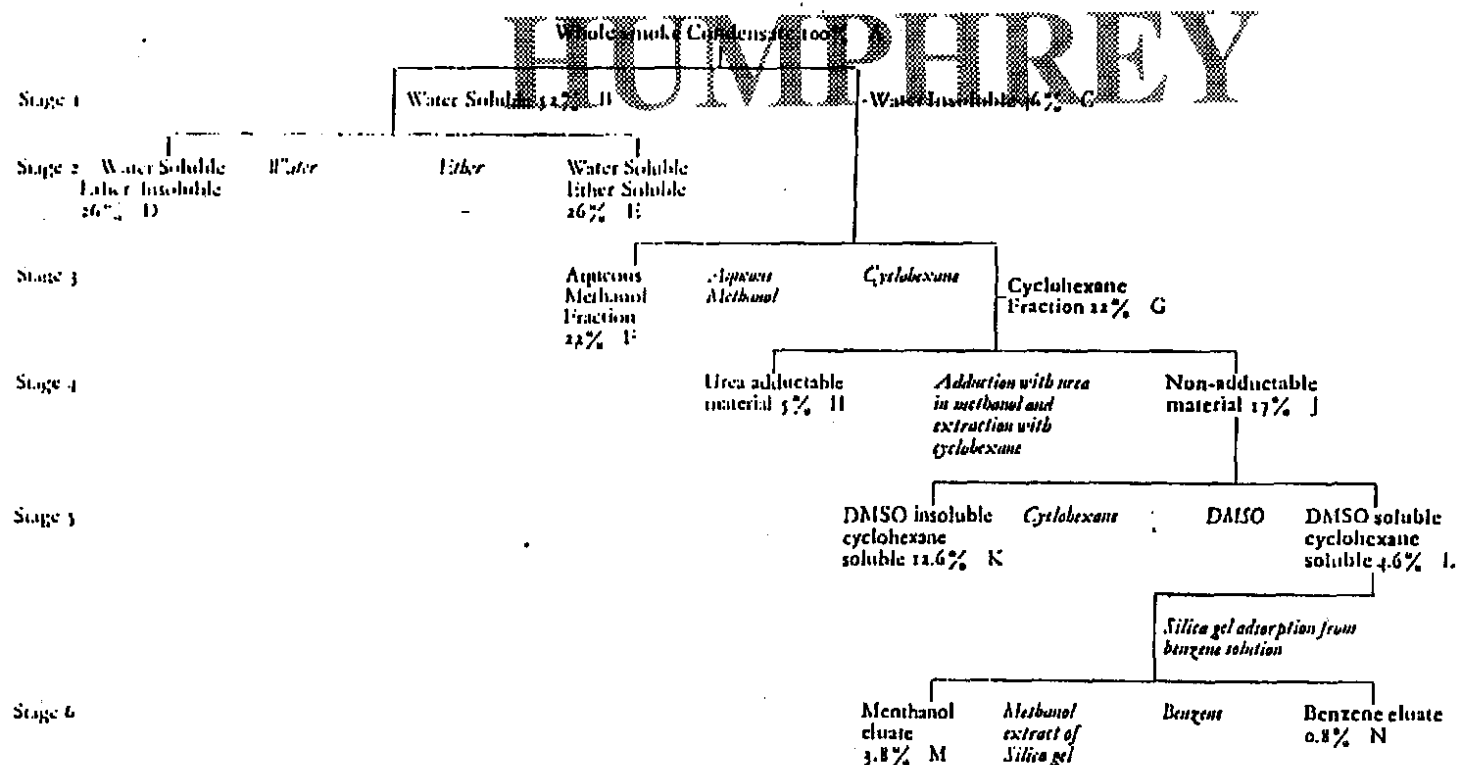
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Diagram 1. Fractionation scheme of cigarette smoke condensate.



Note: 1. The polycyclic hydrocarbons are concentrated into the fractions denoted by heavy outline.  
2. Percentage figures are approximate.

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hydrogen added to whole smoke condensate. Some of these labelled compounds have been synthesised at the Harrogate laboratories.

A smoking machine developed for the Council by the Battelle Institute, Frankfurt, has been installed at the Harrogate laboratories. This apparatus is designed to deliver the whole smoke condensate from four to eight cigarettes dissolved in the appropriate standard volume of acetone for application to the skin of mice within 60 seconds of the production of the smoke. This machine is not yet fully operational. Work will be carried out to determine the differences in chemical composition of smoke condensate produced by this apparatus and a number of other devices designed to produce smoke condensate, and smoke condensate stored for long periods after smoking.

Analyses of constituents of tobacco smoke are carried out where required for specific purposes. This involves not only the routine monitoring of smoke condensate samples used for biological testing and routine analyses, but also the development of analytical methods for specific smoke constituents. The Research Departments of the Member Companies have accepted the main responsibility for the development of standard methods of analysis for a number of smoke constituents. The scientists at the Harrogate laboratories are collaborating in this work.

## Section 2. *Chemical Research by Independent Scientists*

### Possible lactones in smoke condensate

(Dr D. K. Black, Courtauld Institute of Biochemistry, Middlesex Hospital Medical School)

Following the discovery that certain simple alpha-beta-unsaturated lactones were carcinogenic (Dickens and Jones, 1961), a search for such compounds in tobacco smoke condensate was carried out.

The condensate was separated into acidic, phenolic, basic and neutral fractions by conventional extraction techniques. Repeated chromatography of the neutral fraction on alumina and silicic acid and elution with n-hexane and benzene gave a number of unsaturated hydrocarbons: solenesene, farnesene and  $\alpha$ -phthalenes. With the exception of farnesene, all have been reported in the literature. Farnesene was found to the extent of 10mg/1000 cigarettes smoked (Black and Dickens, 1966).

Chromatography of the acidic fraction on silicic acid led to the isolation of levulinic acid (30 mg/100 cigarettes), and since levulinic acid undergoes pyrolytic dehydration to a mixture of alpha- and beta-angelica lactones, the presence of such lactones in smoke condensate might be expected. By addition of labelled lactones to condensate followed by a conventional separation into acidic, phenolic, basic and neutral fractions, it has been observed that a high proportion of the activity was retained by the aqueous phases. Consequently, a study was made of the water soluble fraction of smoke condensate.

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Examination of fractions by gas-liquid chromatography and paper chromatography showed the presence of two hydroxy or keto-acids not detected in the water-insoluble material. However, they were present to an extent too small to be isolated and identified.

The presence of the hydroxycoumarins, scopoletin and esculetin, in tobacco smoke have been reported in the literature and this has been confirmed. Comparative chromatography showed the major fluorescing component to be scopoletin, and a third component has been detected but not identified. Preliminary evidence suggests this is 7-hydroxycoumarin.

In view of the finding that many antibacterial compounds are inactivated by cysteine, Dickens and Cooke (1965) have studied recently the rate of interaction of cysteine with carcinogenic lactones and related unsaturated compounds in an attempt to correlate structure with biological activity, and this work is continuing (Black, 1966).

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### Part 3. PERSONAL, ENVIRONMENTAL AND OTHER FACTORS IN DISEASES ASSOCIATED WITH SMOKING

#### *Section 1. Cardio-Respiratory Disease Research Project*

(Professor D. D. Reid, Professor P. Armitage, Dr J. R. T. Colley, Dr M. Hills, Dr P. M. Lambert, Dr R. J. Prineas, Dr G. A. Rose, Mr B. C. Rowe, Dr G. S. Sorrie, Dr K. Stavrakis and Dr J. A. C. Weatherall, Department of Medical Statistics and Epidemiology, London School of Hygiene and Tropical Medicine)

As reported in the 1963 *Review of Past and Current Activities*, the Council has agreed to contribute up to £500,000 over the 10 years from 1st August 1963 to the London School of Hygiene and Tropical Medicine, so as to enable the Department of Medical Statistics and Epidemiology at the School to be expanded in order to carry out a cardio-respiratory disease research project proposed by Professor D. D. Reid. This research project has four broad aims:

1. to investigate the various environmental factors, such as social, domestic and atmospheric conditions, affecting the onset and evolution of the commoner cardio-respiratory diseases;
2. to identify the physical and psychological characteristics, personal habits and those features of previous medical history which may indicate a special predisposition to these diseases;
3. to study the ways in which general conditions such as air pollution may exaggerate the effects of personal habits such as smoking in the production of cardio-respiratory diseases;
4. to pin-point clinical episodes where smoking may be especially hazardous, e.g. in early stage of coronary heart disease.

The ultimate objective of the project is thus the prevention or reduction of chest and heart diseases. This may require changes in the living conditions contributing to the diseases and identification by various clinical procedures of patients with a special disposition to chest or heart disease so that timely and appropriate action by physicians can be given on a personal basis.

The aims of the project are being pursued by a series of interlocking investigations each designed to answer a specific question but also to illuminate other aspects of the overall problem. These studies should reveal much about the relation of different disease conditions to different personal and environmental factors, and enable the value of these personal and environmental characteristics, as a basis for predicting the later onset of cardio-respiratory disease, to be assessed. It is hoped also to improve current

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methods of identifying susceptible individuals by the application of new clinical methods and modern statistical techniques so as to increase the predictive power of the results of examination and interrogation, and to test the results by a prospective type of field trial. Dr Lambert is now engaged on a detailed analysis of the British data. He and Mr Rowe have developed new methods of using computers to check the internal consistency of replies and of analysing results in large-scale surveys.

As a result of the interlocking approach, the cardio-respiratory disease research project financed by the Council forms an integral and inseparable part of the epidemiological research work of the Department of Medical Statistics and Epidemiology, which is also supported by the Public Health Service of the United States.

*Investigation of external factors in chest and heart diseases*

The results of a survey of chronic respiratory illness carried out in Berlin, New Hampshire, were compared with those in the earlier survey of the College of General Practitioners in the U.K. This comparison showed that the frequency of the simpler form of the disease, with chronic cough and phlegm production, was associated with cigarette smoking and that, once smoking habits were taken into account, there was little difference between the small American town and the rural or urban areas of this country. On the other hand, the severer form of chronic bronchitis with breathlessness and repeated chest illnesses was much commoner, especially among older men, in the large cities of this country than elsewhere in the U.K. or in the American town.

Another survey compared the respiratory symptoms and lung function test results in men between the ages of 40 and 60 doing the same job as van drivers in the postal or telephone services in Central London, in country towns in Southern England and in New York, Baltimore and Washington. Clear indications emerged that some element in the British urban environment, probably air pollution, was responsible for the aggravation of the bronchitis associated with smoking into the severe disease so commonly seen in this country.

The clinical examination of these same occupational groups also covered cardiovascular disorders. Comparison of British and Americans showed that the latter had more electrocardiographic and symptomatic evidence of more severe coronary disease. In these population samples there was no very obvious association between smoking and the prevalence of heart disorder. The higher blood pressures found among the non-smokers could be attributed to their greater fatness.

*U.S.-British-Norwegian Migrant Study*

The Department of Medical Statistics and Epidemiology has been participating with the National Heart and Cancer Institutes of the US Public Health Service and the Norwegian Cancer Registry in a study of cardio-respiratory disease in USA, UK and Norway. At the time of the 1960 Census in the United States, a 25 per cent sample of British and Norwegian immigrants was taken, giving the names and addresses of some 200,000 US residents born in

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Britain and about 30,000 born in Norway. A questionnaire was sent to a representative sub-sample of these (25,000 British-born and 15,000 Norwegian-born), asking information on various subjects, including names and addresses of brothers or sisters resident in the UK and Norway. During the period 1963-65 the relatives of all those between the ages of 35 and 75 of British or Norwegian birth dying in USA were asked as many of the same questions about the deceased person as they could reasonably be expected to answer accurately. In addition, random samples were obtained of native-born men and women over the age of 35 in USA (15,000 in the sample), Britain and Norway.

The questionnaires completed by the samples showed that cigarette smoking was again clearly related to the frequency of persistent cough and phlegm production and to the more serious form of bronchitis with breathlessness and repeated chest illness. The frequency of symptoms of possible heart infarction was related to cigarette consumption, although to a much less degree than the respiratory disorders. In both sexes there was a definite relation between angina of effort and cigarette smoking—a finding at variance with results in some but not all other studies of this point. The frequency of respiratory and cardiac disturbances was related in differing ways to the place of origin (UK or USA; urban or rural) and, in migrants, to the age at migration to the States. British-born migrants to USA come to have the same risk of dying from chronic bronchitis and emphysema as those born in USA. On the other hand, their British childhood is still reflected in an excessive death rate from lung cancer.

The procedures and results to date have been described in papers in *Monograph 19 (Epidemiological Study of Cancer and Other Chronic Diseases)* published by the National Cancer Institute of USA.

#### *Childhood beginnings of bronchitis*

The findings in the Migrant Study and other surveys suggested that some seeds of liability to respiratory disease were sown in youth. The Department began a series of studies of respiratory disorders in schoolchildren. A pilot survey of both upper and lower respiratory tract conditions among over 600 children of both sexes aged 11 years in heavily polluted Sheffield and a like number in the Vale of Glamorgan confirmed the marked social class gradient in the frequency of the severer form of chronic middle ear disease and of chronic sinusitis. There was also a higher frequency within each social class in Sheffield than in Wales. The children in the smokier parts of Sheffield had more attacks of bronchitis and had poorer lung function performance. Of special import was a hint that pneumonia and bronchitis in Sheffield were more likely to result in permanent lung damage than in rural Wales.

#### *Study of chest and heart diseases in families*

The incidence of respiratory disease in children may reflect the effects of personal susceptibility and familial origins whether these be the environment that families have in common or inherited in the strict genetic sense. One of the best ways of distinguishing genetic from environmental factors in disease is by comparison of the illness experience of identical and non-identical

twins, and a collaborative enquiry is being carried out with Dr B. Harvald of the University of Copenhagen who is responsible for the Danish Twin Registry. Preliminary results in twins of both types indicated some excess in heart infarction among the heavy smoking members of the pairs; but the number of deaths was small and a further follow up is now in progress. Other types of family study are in hand in Britain.

*Long-term sickness record in chest and heart disease patients*

Dr K. Szurakky compared the sickness histories of patients suffering from lung cancer over the whole of their working life in the Post Office with those of men of the same age without lung cancer but doing the same job, e.g. as postmen, in the same place at the same time. This comparison showed that lung cancer patients differed significantly in disease experience from the controls for many years before the onset and diagnosis of the terminal condition. They had an excess in bronchitis and other respiratory diseases such as pneumonia, pleurisy and repeated attacks of "influenza". The lung cancer patients had also an excess in peptic ulcer (perhaps because they were likely to be a heavy smoking group), other digestive illnesses, and in rheumatic and joint complaints. The last was a new finding in relation to lung cancer and its significance is obscure.

This study, like an earlier one on the past medical history of chronic bronchitis, suggested that such a use of available records is worth extending to coronary heart disease. Dr R. J. Prineas has made a wide review of the literature on the physical and psychological characteristics believed to be predictors of this disorder, and is now analysing the very full clinical records of some 300 cases of coronary heart disease among men in the Royal Air Force and appropriate controls in the same service.

Dr Rose has produced some evidence from the Post Office studies that the cardiovascular questionnaire used by the Department does predict, to a useful extent, future hospitalisation and death from heart disease.

In the Bedford study initiated by the Medical Officer of Health, Dr Sharp, in collaboration with members of the Department of Medicine at Guy's Hospital, the population was screened by the use of urine and blood tests to identify individuals with diabetes of varying degrees of severity. Dr Rose and others in the Department have been engaged in the cardio-respiratory surveys of these various diabetic groupings. The results showed that people with relatively mild abnormalities of sugar metabolism had more cardiovascular illness than those without them. Smoking was unrelated to these diabetic abnormalities.

Another development in which the Department is closely involved is the trial of mass screening procedures by the Mass Miniature X-ray Unit in Glasgow. Through the help of the Western Regional Hospital Board of Scotland, Dr W. Hawthorne of that Unit has been able to collaborate with the Department in the extension of the more usual aspects of X-ray screening to include the self-administered questionnaires and electrocardiographic and other techniques developed for the GPO and other surveys. The aim is to adapt these procedures to cope with the practical problem of screening large numbers of men in a short time. Dr Sorrie and Dr Prineas have been working

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closely with Dr Hawthorne on the development of radiological and electro-cardiographic methods of detecting the early stages of cardio-respiratory disorder.

#### Other special studies

There are good reasons for investigating the influence of smoking and other factors at times of special physical stress.

Professor Reid is making a special study of smoking habits in relation to the progress of patients admitted to the Medical Research Council Trial of Anticoagulant Therapy for patients suffering from heart infarction. Dr J. A. C. Weatherall surveyed the deaths and hospital statistics which showed that pulmonary embolism and other thrombo-embolic disorders were rising steadily in adults of both sexes. She concluded that this rise was unlikely to be attributable to changes in smoking habits. Dr Rose has, with members of the Department of Obstetrics at St Mary's Hospital, been making regular and precise measurements of blood pressure during the whole course of pregnancy and recording current smoking habits and the outcome of the pregnancy to see how far they are inter-related. Professor Armitage and Dr Hills have developed for use in these studies sophisticated methods of statistical analysis which should prove invaluable in collating results and improving the efficiency of prediction of premature disease or deaths based on data obtained by the clinical procedures.

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## Section 2. Lung Cancer and Bronchitis

### 1. Lung cancer and bronchitis mortality in Northern Ireland (Dr G. Dean and Mr A. J. Wicken)

An epidemiological study of the lung cancer and bronchitis deaths of men and women aged 35 or more that occurred during the years 1960-62 in Northern Ireland was organised by Dr G. Dean. Mr A. J. Wicken of the Health Surveys Unit of AGB Research Ltd supervised the statistical and interviewing work involved in the study. The cost of the enquiry was met by the Council.

The lung cancer mortality rates of both men and women aged 35 or more in Northern Ireland were substantially below those in England and Wales. So was the bronchitis mortality rate of men, but the bronchitis mortality rate of women aged 35 or more was almost the same as that in England and Wales. Chronic bronchitis was certified as a contributory cause of about one-third as many deaths as were directly caused by it. About one-third of all death certificates of men in central Belfast had the word "bronchitis" on them.

The lung cancer mortality rate was found to be associated with smoking habits, area of residence, social class, air pollution of occupation and morning cough three or more years before death. The chronic bronchitis mortality rate was found to be associated with area of residence, smoking habits and, to a less extent, social class. Lung cancer mortality was more closely associated with smoking habits than with the presence or absence of morning cough, more closely with morning cough than with area of residence, and more closely with area of residence than with social class. The bronchitis mortality rate was associated to an approximately equal extent with area of residence and smoking habits, and to a less extent with social class. Among male non-smokers, the lung cancer and bronchitis mortality rates were both between three and four times higher in Belfast than in truly rural districts. The lung cancer rate of male non-smokers in truly rural districts of Northern Ireland was very low.

The enquiry has been completed and reports on it by Dr Dean and by Mr Wicken have been published.

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Wicken, A. J. (1966). Environmental and personal factors in lung cancer and bronchitis mortality in Northern Ireland 1960-62. *TRC Research Paper No. 9*.

### 2. Lung cancer and bronchitis mortality in areas of North-East England

(Mr A. J. Wicken and Dr S. F. Buck)

At the invitation of the Health Committee of the Urban District Council of Eton and with the co-operation of the other Health Committees concerned.

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an investigation was undertaken into certain environmental factors which seemed likely to be associated with lung cancer and bronchitis mortality in Eston, Stockton-on-Tees and the four rural districts of Croft, Northallerton, Richmond and Stokesley, Yorkshire. Mr A. J. Wicken and Dr S. F. Buck of the Health Surveys Unit of AGB Research Ltd organised the survey. Information was obtained about the age, smoking habits, occupation, previous respiratory illness and exposure to air pollution of all men and women aged 35 or more who had died from lung cancer or bronchitis during the years 1962-67. The level of air pollution in three sites in Eston was measured each week for a period of twelve months.

The study showed that the lung cancer mortality rates of men and women were distinctly greater in Eston and Stockton-on-Tees than they were in the four rural districts. At the end of the period, the male lung cancer rate was well above the national average in Eston, equal to it in Stockton and well below it in the four rural districts. The bronchitis mortality rates for both men and women were distinctly greater in Eston than in Stockton-on-Tees and greater in Stockton than in the rural districts. At the end of the period, both male and female bronchitis mortality rates were well above the national average in Eston, slightly above it in Stockton-on-Tees and well below it in the rural districts. Statistical associations were found between smoking, social class, area of residence, air pollution at work and mortality from lung cancer and bronchitis. An association between previous illness from bronchitis and lung cancer was also found. In the areas covered, lung cancer mortality appeared to be more strongly associated with smoking habits than with the level of air pollution, but the ranking of these two factors was reversed for bronchitis mortality. The results of the enquiry were published as TRC Research Paper No. 8.

#### References

- Wicken, A. J., and Buck, S. F. (1964). Report on a study of environmental factors associated with lung cancer and bronchitis mortality in areas of North East England. TRC Research Paper No. 8.

#### 3. Models for use in investigating the risk of mortality from lung cancer and bronchitis (Dr S. F. Buck and Mr A. J. Wicken)

A large number of epidemiological investigations, including those supported by the Council, has shown that individuals with certain personal characteristics and exposed to certain environmental factors had a much greater risk than others of dying from lung cancer or bronchitis. In these circumstances, relatively small groups of people with relatively great risks of dying from lung cancer or bronchitis might make a major contribution to mortality from these diseases. It was considered that if such groups could be identified, it might be possible to take measures to reduce their risk. As background information for any advance along these lines in preventive medicine, the Health Surveys Unit of AGB Research Ltd was asked by the Council to construct models from the data accumulated in the epidemiological studies

in Northern Ireland and in areas of North East England for use in investigating the risk of death from lung cancer or bronchitis.

Discriminant analysis techniques were used in order to identify the factors which, both separately and when taken together, discriminated most effectively between lung cancer or bronchitis subjects and their respective controls. The factors thus identified as being most closely associated with mortality from lung cancer or bronchitis were built into multiplying models. These models consisted of (1) a basic mortality rate for men who did not have any of the characteristics studied (i.e. they were men who did not smoke, did not cough, lived in rural areas, etc) and (2) estimates of the effect which each factor then had in multiplying the basic mortality rate.

The results will be published.

#### 4. Smoke and sulphur dioxide in relation to lung cancer and bronchitis mortality

(Dr S. F. Buck and Mr D. A. Brown)

A statistical investigation into mortality from lung cancer and bronchitis in relation to smoke and sulphur dioxide concentration, population density and social index was carried out by Dr S. F. Buck and Mr D. A. Brown of A.G.B. Research Ltd. The investigation covered 219 districts in England and Wales for which comparable data was available. Smoke and sulphur dioxide figures were taken from the national survey of air pollution started in 1960 by the Department of Scientific and Industrial Research with supplementary figures for other areas.

The results were published in TRC Research Paper No. 7. Differences in the mortality rates from lung cancer between different districts of the same administrative type could not be accounted for by differences in smoke and/or sulphur dioxide concentrations in residential areas of these districts. The differences in mortality were, however, closely related to differences in population density. Differences in mortality from bronchitis between different districts of the same administrative type were associated with variations in the concentration of smoke and of sulphur dioxide in the residential areas of these districts.

The differences in consumption per head of cigarettes between urban and rural areas were also examined. It had been stated that smoking may have spread from town to country and that the lower lung cancer mortality rate in rural districts may have reflected lower cigarette consumption in the past. If this hypothesis was correct, urban and rural lung cancer mortality rates would be tending to converge. The investigation showed that this had not happened up to 1961.

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#### 5. Lung cancer in the Channel Islands (Dr G. Dean)

Lung cancer in the Channel Islands is high and Dr Dean carried out an investigation of the place of birth and smoking habits of all men and women who had died from lung cancer in the Channel Islands during the years 1951-61 and of a control group. Information was sought about 301 deaths and 100 per cent response was obtained.

Over the period 1951-61, UK born men who had become resident in the Channel Islands did not have a significantly different lung cancer mortality rate from locally born men. During the last few years, however, a trend of earlier years had been reversed and the lung cancer mortality rate of British immigrants now exceeded that of locally born men. The difference between cigarette consumption per head of British immigrants and locally born men in the Channel Islands was not statistically significant. The lung cancer mortality rate for locally born men and British immigrants combined was significantly higher in Jersey than in Guernsey, Alderney and Sark. British born men who settled in the Channel Islands smoked more cigarettes than men in England and Wales but did not have a significantly different lung cancer rate from the latter. The results of the study have been published.

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#### 6. Lung cancer in rural South Africa (Dr G. Dean)

Dr Dean had carried out two studies of lung cancer among white South Africans (Dean 1959, 1961) and a supplementary study of lung cancer among white South African born men living in rural areas (Dean, 1961). These studies had been financed by the Council. As a relatively high rate of non-response had been encountered in these studies, the Council agreed with a recommendation by Dr Dean that a further effort should be made to obtain an improved response rate in the rural areas and to bring the results up-to-date. In this study, Dr Dean received the assistance of SANLAM, a large South African life assurance company, in tracing the relatives of those who had died. As a result of this further search, the response rate in rural areas was increased from 77 per cent to 81 per cent.

Revised lung cancer mortality rates at each level of smoking for the period 1947-60 were calculated, on the basis of the larger numbers of deaths obtained, for white men aged 45-64 born in South Africa and resident in rural areas. The inclusion of the additional 55 lung cancer decedents whose relatives had been traced made only slight differences to the lung cancer mortality rates which had been calculated from the earlier figures. In particular, the relatively low lung cancer mortality rate of white men aged 45-64 born in South Africa who smoked 1-20 cigarettes per day (15 deaths per 100,000 per annum) was almost the same as that in the earlier enquiries (13 per 100,000). The very low lung cancer mortality rate previously estimated

for non-smokers in this group (7 deaths per 100,000 per annum) was reduced to a rate of 5 deaths per 100,000 per annum. The results of this follow-up study have been published (Dean, 1965).

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#### 7. Bronchitis in relation to lung cancer

(Dr E. N. Davey, Dr W. E. H. Field, Dr L. Reid and Dr F. J. C. Roe, Gloucester and London)

This project consists of two parts. The object of the first part was to ascertain, by epidemiological and pathological methods, the status of the population served by certain hospitals in the Gloucestershire area with regard to the clinical and preclinical stages of chronic bronchitis. Pathological assessment of bronchitis status included the use of various measurements of mucus-producing capacity. The object of the second part was to compare the bronchitic status of lung cancer patients and matched controls in order to throw light on the relation between lung cancer and bronchitis and to test the hypothesis that chronic bronchitis predisposes to lung cancer. A report on the analysis of the material obtained up to June 1964 has been published (Field *et al.*, 1966). The main findings were that the two indices of mucus gland hypertrophy used, "the Reid index" and "acinar count", were reproducible for any one case and related to each other to a highly significant degree. In addition, both indices were correlated with a history of chronic cough and sputum production.

The proportion of males with mucus gland hypertrophy was found to increase with age, particularly in smokers. Pipe smokers and ex-cigarette smokers had almost as high an incidence of mucus gland hypertrophy as cigarette smokers.

No association was found between residence (i.e. urban or rural) and mucus gland hypertrophy, but it is likely that extremes of high or low air pollution are not found in the Gloucestershire area.

The findings in women differed markedly from those in men in that smoking appeared to have relatively little effect on the mucus gland. The only significant difference found in females was between urban cigarette smokers and rural cigarette smokers. This difference was significant only in the case of one of the two indices, and was in the direction of more hypertrophy in the urban group.

Work on the second part of the main project is still in progress.

#### Reference

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### 8. Study of respiratory diseases

(Dr S. Carne, College of General Practitioners, London)

During the winter of 1962-63, Dr S. Carne of the Research Section of the College of General Practitioners, in conjunction with the Medical Officers of Health of the London Boroughs and the Department of Scientific and Industrial Research, organised a survey of the effects of air pollution on respiratory illness in the population of London and Sheffield during the 26 weeks from October 1962 to April 1963. Each participating general practitioner completed a daily record sheet for each consultation, recording in particular whether a consultation was for a respiratory disease.

A further study was carried out during the winter of 1963-64 and the Council made a grant to the College of General Practitioners towards the cost of analysing the results of the survey.

A preliminary report of the findings from the first winter was published in the Proceedings of the Royal Society of Medicine. A report incorporating some of the results from both winters was presented at the International Clean Air Conference in London in September 1966. A final report is in preparation.

#### Reference

Carne, S. (1964). *Proc. R. Soc. Med.*, 57, 620.

### 9. Smoking and airways resistance

(Dr C. M. Fletcher and Dr B. Clarke, Royal Postgraduate Medical School, London)

Disability in bronchitis is chiefly due to increased airways resistance. McDermott and Collins (1965) showed that using the whole body plethysmograph technique (Dewhurst *et al.*, 1956) bronchitic subjects had a greater increase of airways resistance on smoking one cigarette than normal subjects. Dr C. M. Fletcher and Dr B. Clarke are developing this work with a view to assisting identification of individuals sensitive in this way to the smoke of one cigarette and to discovering which fraction of the cigarette smoke is chiefly responsible for this reaction. The Council has supplied in addition to financial support for this work, standard cigarettes, filter cigarettes with reduced smoke vapour phase and Cambridge filters to eliminate smoke particulate phase so that some indications may be obtained of the broad groups of smoke constituents that may act to produce increased airways resistance in sensitive persons. Both particulate and vapour phase filters have been shown to reduce the bronchial reaction to smoke. Further studies are proceeding.

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## 10. Exfoliative cytology of the respiratory tract

(Professor C. V. Harrison, Dr S. W. A. Kuper and Dr P. Stradling, Royal Postgraduate Medical School, London)

Tumour cells have been shown to have reduced mutual adhesiveness, so it seemed reasonable to hope that exfoliative cytology would help patients by establishing the diagnosis earlier than could be expected by more conventional methods of investigation. Over 1,500 sputum samples have been examined and of these over 170 were found to have tumour cells. These positive samples came from over 80 patients, in the majority of whom the diagnosis had not been definitely established, despite intensive investigation. Real assistance in the investigation of these patients has been achieved and this has resulted in a greatly increased demand for the use of the method.

In the earliest phases of malignancy one cannot hope to find exfoliated cells as easily as in the later fungating growths. Cellular abnormalities are likely to be less striking, and numbers to be small. Because of these considerations, it was considered essential to develop more sensitive cytological techniques, and if possible quantitative ones. After much experimental trial and error, two such techniques are now being developed, viz:

- (a) Liquefaction of sputum followed by centrifugation and preparation of "issue" sections was undertaken in the expectation that efficient liquefaction would allow the differential centrifugation of cellular elements from sputum, and as tumour cells usually have a lower specific gravity than normal cells, it was hoped to concentrate the tumour cells in the upper strata of the button of cells. It was imperative that cells should not be damaged morphologically nor in respect of their staining characteristics. The mucolytic method used is a combination of oxidising agents and an ultrasonic disintegrator. The latter is able to shatter completely the strands of mucoprotein, which do not then impede the cells during centrifugation. The cell buttons thus liberated are processed by conventional histological methods, and sections cut and stained. A summary of this method was reported at the 9th International Cancer Congress (Tokyo).
- (b) The presence of cancer is sometimes predicted from sputum examination in patients with no localising X-ray shadow. In such patients the localisation of the tumour has till recently been virtually impossible, and it became urgently necessary to devise a technique for sampling the separate lobar bronchi. Such a method should also be able to find cancer cells with greater sensitivity and therefore earlier than is possible with sputum derived from the whole chest. A method has only recently been developed, but it has already been of value in establishing diagnosis.

It is hoped to use the two techniques described, together with ordinary sputum cytology, in the investigation of patients to establish early diagnosis. After further elaboration the methods will be subjected to thorough comparative trials. Having established the technical reliability of the methods, it is planned to undertake a progressive clinical survey to ascertain whether

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early pre-malignant changes in the respiratory tract can be detected.

#### Reference

Kuper, S. W., A. Stradling, P., Davis, J., and Shortridge, D. (1966). *Lancet*, 2, 982.

#### 11. Neuraminic acid and bronchitis

(Dr L. Reid and Dr E. E. Keel, Institute of Diseases of the Chest, Brompton Hospital, London)

The early stages of chronic bronchitis are now defined as excessive production of mucus from the bronchial tree, which is known to be accompanied by hypertrophy of the bronchial mucus glands, particularly of acini containing acid mucopolysaccharide. The object of the present study was therefore to investigate mucus production in chronic lung disease using a sialic acid found in the mucus, namely N-acetyl neuraminic acid, as a marker.

Since uncontaminated bronchial secretion is rarely available, it was necessary to use sputum and the contribution of saliva to the total sialic acid content had to be assessed. The level in saliva was found to be about 25 per cent of that in bronchial secretion when expressed as a percentage of dry weight, but since the amount of dry material obtained from saliva is small it contributes less than 5 per cent on a weight/volume estimation.

Serial specimens of sputum from 48 patients over a period of 2½ years have been analysed for sialic acid content and have shown a pattern of seasonal variation. This is not related to exacerbations of bronchitis or to the presence of pathogenic organisms and further studies are envisaged to investigate the relation to atmospheric pollution.

Absolute values of sialic acid have shown good correlation with a clinical assessment of sputum viscosity. No relationship has been demonstrated between sputum viscosity and duration of disease, age or occupation of patient, volume of sputum or smoking habits.

Sputum samples have also been examined from patients with bronchorrhea, with cystic fibrosis, and from 25 patients about to undergo lung resection at which a ring of bronchus has been obtained for histochemical and tissue culture studies. These studies suggest that sialic acid may be linked to the fundamental structure of bronchial mucus in at least two ways, the one susceptible to enzymatic degradation with neuraminidase from the cholera vibrio and other splitting only with acid hydrolysis. A quantitative difference in sputum has been shown and some correlation seen with the staining reaction of the bronchial mucus glands in tissue section.

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## 12. Psychosomatic factors in relation to lung cancer

(Dr D. M. Kissen, Psychosomatic Research Unit, University Department of Psychological Medicine, Southern General Hospital, Glasgow)

The Psychosomatic Research Unit is concerned with psychosomatic aspects of cardio-respiratory disease. The main project has comprised studies of lung cancer in men. Since the establishment of the Unit, its work has been extended to the psychophysiological field and to possible correlations of personality with adreno-cortical function, with particular reference to the development of lung cancer.

As a result of clinical observations (made without knowledge of diagnoses) on patients admitted to the chest units of three hospitals in the Glasgow area, carried out since 1958, Dr Kissen formed the hypothesis that lung cancer patients more frequently than non-cancer patients had what he termed "poor outlets for emotional discharge". This he defined as a facility for absorbing, and an unconscious containment of, emotional conflict without apparent effective overt or covert response. Dr Kissen found that lung cancer patients had a significantly lower average score for neuroticism than controls on the short form of the Maudsley Personality Inventory. This inventory was chosen because it measures two dimensions of personality, neuroticism and extraversion, relevant to problems being considered. Neuroticism, in the context of this study, is defined as the individual's emotional lability, emotional over-responsiveness and liability to neurotic breakdown under stress. Dr Kissen predicted therefore that lung cancer patients would have low neuroticism scores which would reflect low emotional lability, emotional under-responsiveness, etc. and considered that such findings would be consistent with his hypothesis. The other measure of the MPI, extraversion, had been reported as a feature of cigarette smokers. The association between lung cancer and a low average score for neuroticism was found at all levels of cigarette smoking; and among pipe smokers there was only a small exception in one group. The association between low neuroticism score and lung cancer was less, however, than the association between heavy cigarette smoking and lung cancer. It was not related to the histological type of tumour developed by lung cancer patients. Lung cancer patients were also found to have a significant tendency to conceal emotional problems. They also gave a frequent past history of certain psychosomatic disorders. In these latter findings lung cancer patients showed a parallel with those non-cancer patients who presented with, or gave a history of, psychosomatic disorder.

Dr Kissen also found that lung cancer patients had a significantly lower level of reporting of certain childhood behaviour disorders than controls, and this had a strong statistical correlation with low neuroticism scores. He considered that this finding provided further support for his initial hypothesis.

There was not a significantly greater degree of extraversion in lung cancer patients than in controls though there was a slight trend in this direction.

Dr Kissen has also reported that lung cancer patients who smoked cigarettes but said they did not inhale had lower average neuroticism scores than lung cancer patients who said they were inhalers. He suggested that the poorer the outlets for discharge of emotion, the less was the exposure to cigarette smoke

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required to induce lung cancer. In addition, those with poor emotional outlets (in terms of low average neuroticism scores) were estimated to have four and a half times the lung cancer mortality of those with good emotional outlets. Lung cancer patients were also estimated by him to give a significantly frequent past history of certain psychosomatic disorders in close relatives, including wives, a feature which they have in common with non-cancer psychosomatic patients.

Recently Dr Kissen has published psychosocial findings in relation to lung cancer in men aged 51-64. These concerned childhood where the key factor appeared to be separation from parental unity (especially death of a parent or unhappy home due to chronic friction between parents), and adult life where significant adverse events appeared to be related to work and interpersonal relationships, especially where these were of long duration. Lung cancer groups with and without a history of adverse life situations both showed similar low average neuroticism scores. In contrast, non-cancer groups with a history of adverse life situations showed significantly higher neuroticism scores than those with no such history. These findings Dr Kissen considered consistent with the hypothesis that lung cancer patients have poor emotional outlets. He emphasised the importance of the interplay between life situation and personality in determining the impact on the individual of these adverse life situations.

The differences between the lung cancer patients and controls were found in studies in which patients answered questions asked in interviews and wrote answers to typed questionnaires. The controls were usually age-matched chest patients diagnosed to have some disease other than lung cancer. At the conclusion of the interview, patients were asked to complete the MPI. Dr Kissen has considered the possibility that the differences between the answers to the MPI given by lung cancer patients and controls might have been due to many of the former suspecting that they had lung cancer. He has rejected this suggestion for two reasons. Firstly in no case was any diagnosis known to him, nor had a diagnosis of cancer been given to any patient by the time of interview. Secondly the evidence suggested to him that suspicion of a diagnosis of cancer was more likely to result in raised than lowered N scores. He reached this conclusion from his daily experience with patients and from the results of a study of some lung cancer patients who had already undergone deep X-ray therapy or lung resection and who were found to have markedly higher N scores than patients with no surgical procedure. Most of the group who had undergone surgical or radium treatments must have suspected from the nature of these measures that they had cancer. Since all the studies reported were retrospective, Dr Kissen does not exclude the possibility that the disease process itself may have influenced findings. His research has therefore been designed with a view to the possibility of prospective studies.

Dr Kissen is aware that the early measures of personality used by him lack sufficient precision and stability to discriminate cancer-prone individuals although they are reasonably successful in discriminating cancer and non-cancer groups, and he has drawn attention to the inconsistency of neuroticism scores with variable environmental conditions (using the short form of the MPI). Dr Kissen hopes that other measures presently being pursued may

provide better delineators of personality. He has emphasised the potential value, in his opinion, of the psychosomatic approach in the prevention of occurrence and recurrence of cancer.

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#### Section 5. Cardiovascular disease

1. Morphology and pathogenesis of atherosclerotic plaques  
 (Dr S. Woolf and Dr K. Carstairs, St George's Hospital Medical School, London)

The application of Coons fluorescent antibody technique to a study of the distribution of lipoproteins within the arterial intima shows that beta lipoproteins are present in a variety of patterns of localisation in atherosclerosis. The results appear to support the hypothesis that, in uncomplicated lesions, the lipoprotein is derived from circulating plasma as a result of a filtration process (Woolf and Pilkington, 1965).

During this study lipoproteins were observed in small mural thrombi occurring in relation to atherosclerotic plaques. This observation was followed up by a systematic immunohistochemical study of thrombi of varying ages and occurring in different situations within the vascular system. Lipoprotein has been identified in most of the thrombi studied and if intensity of fluorescence has any quantitative implications, mural thrombi appear to contain more lipoprotein than occlusive thrombi. Artificial thrombi prepared from platelet-rich plasma also contain lipoprotein, so it is likely that the lipoprotein is incorporated within the thrombus structure at the time of platelet aggregation and fibrin deposition. These results have interesting implications in so far as they suggest a means whereby mural thrombi may contribute to plaque lipid content (Woolf, Pilkington and Carstairs, 1966).

Another application of the immunohistochemical techniques to vascular tissue has been a study of the characteristic glomerular lesions in the kidneys of diabetic subjects. Fibrin and lipoprotein have been demonstrated in these lesions though attempts to visualize other plasma proteins have failed. The results suggest that there may be a selective hold up of plasma proteins

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filtering through the glomerulus wall in a manner analogous to what the authors have suggested occurs in the process of early plaque formation (Davies, Woolf and Carstairs, 1966).

The authors suggest that by immunological means it might be possible to determine the relative contributions of mural thrombosis and infiltration of plasma lipids and proteins in plaque growth. During the past year this work has continued using sera prepared against human fibrinogen and human platelets. This study has now been completed. The results so far analysed suggest that it may be possible to differentiate on the basis of varying distribution patterns of fluorescent material between plaques in which a thrombotic element is present, and those where plasma proteins have gained access to the vessel wall by a process of infiltration.

The technique of thin-layer chromatography is being applied to frozen sections of intact artery blocks. This method allows the characterisation of the lipid content of individual lesions at all levels within the walls, and studies are proceeding on the lipid and protein changes in various types of atherosclerotic lesions, and also in macroscopically normal portions of the vascular tree which are "high-risk areas" for the development of atherosclerosis (Lidzky and Woolf, in the press).

The immunohistochemical work carried out in all types of atherosclerotic lesions up to the present suggests that there may be a selective "hold-up" of the larger molecules—fibrinogen and beta lipoproteins. Anti-sera against human albumin and human  $\gamma$ S gamma globulin have been prepared and studies are continuing to see whether these proteins can also be visualised in atherosclerotic intima. Absence of albumin and gamma globulin would support the hypothesis that only part of the infiltrating stream of plasma is held up within the arterial wall.

The study is continuing.

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## 2. Dietary effects in the formation of atherosclerotic plaques and thrombi and the effects of nicotine on fat metabolism (Professor T. Crawford, St George's Hospital Medical School, London)

Since 1946, much attention has been focused on the role of thrombosis as an important factor in the development of atherosclerosis, and it seems likely that mural thrombosis contributes significantly to the growth of atherosclerotic plaques, especially in the coronary circulation. Similarly, careful inspection of the inner lining of a large artery such as the aorta reveals the presence of small thrombi on parts of the wall unaffected by atherosclerosis. It is not yet clear, however, what the significance of these fine encrustations is and whether they form a nidus for the development of future atherosclerotic lesions.

Many studies have been undertaken to find an answer to this question and most have been based on the injection of blood clots or an artificial thrombus into the pulmonary circulation of experimental animals. These circulating masses become impacted in branches of the pulmonary arteries and later form eccentric thickenings on the vessel wall, which clearly show that, in certain circumstances, thrombus material may be converted to lesions resembling to a degree atherosclerotic plaques. The extrapolation of these results to human disease can, however, be misleading since occlusion of the affected vessels at some stage leads to a different reaction from that elicited by mural thrombi. Further, the dynamics of blood flow in the pulmonary circulation differ markedly from those in systemic arteries such as the aorta or coronaries, and the rabbit, which has been the most frequently used animal in these experiments, does not spontaneously develop atherosclerosis and has a natural dietary regimen which renders alterations in its blood lipid levels difficult to effect without unphysiological manoeuvres.

The present study is supporting Professor Crawford and his colleagues in a study involving the production of mural thrombi in the abdominal aorta of the pig and following the natural history of this lesion for up to two years. The pig has been chosen because it is omnivorous and has serum lipid levels not very different from those found in humans. It also is an animal which spontaneously develops atherosclerosis with advancing age and the lesions closely resemble those found in man. It has the added advantage that the serum lipid pattern can be manipulated by dietary variations, which are comparable with those found in different human populations.

A pilot study undertaken to explore different surgical methods of producing mural thrombi has shown that aortotomy followed by gentle trauma to the intima under direct vision produces the most consistent results. This method has been adopted for the main study and has produced thrombi in 100% of the animals sacrificed three days after operation.

It is hoped to discover whether alterations in the serum lipid levels cause changes in the incidence, size, or in the natural history of mural thrombi. This is of particular interest in view of the correlation of elevated serum lipid levels in man with coronary thrombosis. In addition the effects of substituting a low-fat diet for a high-fat diet on the blood lipids themselves are well known. There is, however, little or no knowledge of the effects of such dietary manipulations on thrombi and a demonstration that low-fat diets or a high intake of unsaturated fatty acids reduces the incidence and extent of mural thrombosis or hastens the regression of the lesions would obviously be of great importance.

An interesting by-product of the main study has been the discovery of an unusual reaction to the depolarizing muscle relaxant suxamethonium chloride which appears to be genetically determined (Hall *et al.*, 1960).

Further genetical and pharmacological studies are being undertaken.

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### 5. Lipids in arterial disease

(Professor R. Mahler, Department of Clinical Chemistry, Welsh National School of Medicine, Cardiff; Dr T. Csorba and Dr M. Root, Guy's Hospital Medical School, London)

In coronary heart disease there are fatty deposits in the walls of the coronary arteries which bulge into the lumen of the artery, disturbing the flow of blood through it and making it more liable to be completely blocked by a thrombus. The study, which is being supported by a grant from the Council, has as its objective the definition of the factors involved in the accumulation of lipids in the arterial wall. It is being approached from two directions. Firstly, a study of the factors which determine the synthesis and deposition of lipids, and secondly, a study of the factors which control the breakdown and removal of lipids from the arterial wall. These two processes balance one another in a normal artery, but if synthesis and deposition of fat become too rapid, or if the breakdown and removal of fat become too slow, it begins to accumulate in the wall of the artery.

With regard to the synthesis and deposition of fat in the artery, it has been shown that there is a correlation between the level of triglyceride fat in the blood and the degree of arterial damage, and that the triglycerides from the blood can enter the arterial wall and be deposited in its inner layers. Therefore, factors which can lead to increased blood triglyceride levels, such as a high level of ~~cholesterol~~ or fructose in the diet, may precipitate damage to the arteries. The levels of triglycerides and fatty acids are also raised when there is active mobilisation of fat from adipose tissues, as occurs in badly controlled diabetes, or is stimulated by adrenaline and other hormones.

In connection with the removal and breakdown of fat in the artery, the initial step is controlled by a fat-splitting enzyme which Professor Mahler has shown to be present in the cells of the inner coats of the arterial wall. In human arteries and in the arteries of several animal species, the activity of this enzyme is influenced by sex and age. The fat-splitting enzyme is most active in women before the menopause, it is less active in men of the same age and is very low in the arteries of old men and women. This observation agrees well with the incidence of arterial disease in the population and suggests that the lipolytic enzyme plays an important role in the control of fat accumulation in the artery.

A detailed study has been made of the effect of nicotine and related compounds on the activity of the lipolytic enzyme in the artery and in adipose tissue. At high concentrations, nicotine and some of its analogues are potent inhibitors of the arterial enzyme, but have no effect on the enzyme in adipose tissue; other analogues, like coranine, have no effect on either enzyme. At low concentration, however, some of the analogues, although not nicotine itself, have a slight stimulatory action on the arterial enzyme only. Nicotinic acid, which is a well-known inhibitor of the fat-splitting enzyme in adipose tissue, also inhibits the arterial enzyme at high concentrations; but at lower concentration, when it has no effect on the adipose tissue enzyme, it significantly stimulates the fat-splitting enzyme in the artery.

These studies have led to a search for other compounds which could

increase the activity of the fat-splitting enzyme in the artery, without stimulating the enzyme in adipose tissue, and so avoiding the rise in fatty acids and triglycerides in the blood which would follow the mobilisation of fat from adipose tissue stores.

#### 4. Metabolism of lipids in normal and atherosclerotic arteries of non-human primates

(Mr D. E. Bowyer, Department of Pathology, University of Cambridge)

Atherosclerotic lesions may be induced in experimental animals by feeding diets rich in fat. For example, this has been achieved in baboons given a diet containing egg yolk and butter and the lesions obtained are histologically similar to fibrous human lesions. It has also been shown that the changes in lipid composition produced in the arteries of these animals are similar to changes found in atherosclerotic human arteries. Studies involving such a system are being supported by the Council and should lead to more knowledge of the events taking place during the metabolism of lipids in normal and atherosclerotic tissue. Further objects of this work are to elucidate the role of humoral factors such as glucose and hormone levels in the mechanisms of synthesis and deposition of arterial lipids in atherosclerosis. The results which it is hoped to obtain should ultimately be of value in increasing understanding of the biochemical abnormalities in atherosclerosis and coronary heart disease.

#### 5. Metabolic defects in the arterial wall in relation to coronary and cerebral ischaemic disease

(Professor C. W. M. Adams, Guy's Hospital Medical School, London)

Professor Adams and his colleagues are receiving support from the Council in studies concerned with the metabolic processes for disposing of lipids in the arterial wall. One of the hypotheses in which these workers are interested is that an increase of the arterial phospholipid level may stabilize the suspension of cholesterol in the vessel and thus prevent its deposition in the arterial wall.

Another aspect of this research involves the possible protective action of the lipolytic enzymes of the arterial wall. Thus, previous histochemical and biochemical studies indicated that there is little triglyceride accumulation in atherosclerosis. However, further studies have shown that there is a slight increase in triglyceride levels with ageing, which is matched by a corresponding decline in lipolytic activity. At present sex difference in aortic lipolytic activity is being investigated.

Other enzymes involving the esterification and transacylation of cholesterol in arterial tissue have been examined and a cholesterol transacylase has been identified in the aorta. This enzyme uses unsaturated phospholipids as a fatty acid donor. It is particularly relevant to this observation that polyunsaturated phospholipids cause much faster resolution of cholesterol implants than does saturated phospholipid.

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Studies involving the use of electron microscopy have been carried out on the intracellular localization of lipid deposits and of lipid synthesis in the arterial wall.

## 6. Blood coagulability and platelet adhesiveness in relation to atheroma

(Professor H. W. Fullerton, Dr N. B. Bennett, Dr P. Bennett, Dr A. A. Dawson, Dr D. Ogston, and Dr M. Ogston, University of Aberdeen)

This group of workers is studying certain aspects of the mechanism which results in the clotting of the blood and how this might alter due to the interplay of several factors. The research falls under three main headings which are fibrinolysis, platelet adhesiveness and a factor which clears lipid from plasma.

### *Fibrinolysis*

This is the process by which the structural proteinaceous material of a blood clot is enzymically degraded to soluble products. It is a very complex process which involves several interlocking reactions. Thus an inactive globulin occurring in the blood plasma, namely plasminogen, is activated under suitable circumstances to an enzymically active protein, plasmin. Plasmin can then bring about the degradation and solubilisation of fibrin and dissolution of a fibrin clot. The activation of plasminogen is brought about by substances, termed activators, which occur in blood, urine and in body tissues. These activators can be counteracted in their ability to catalyse the formation of plasmin from plasminogen by certain inhibitory substances. Active plasmin can itself also be inhibited and there are therefore two separate parts of the system which might be restrained.

In attempts to understand this system more fully, several factors which have an effect on the process of fibrinolysis have been studied. It has been shown that differences occur between men and women which are presumably at least in part due to hormonal causes since it can be shown for example that the administration of oestriol to men decreases significantly the level of anti-plasmin in the blood. This may partially explain the lower susceptibility of women of reproductive age to atheroma and thrombosis. After the menopause the susceptibility of females rapidly approaches that in males of the same age.

Following myocardial infarction, where the blood supply of part of the heart muscle is interfered with, or after surgical trauma, there is an increase in plasma volume and changes in plasma plasminogen similar to those which occur after injury. It is thought that the changes which are observed are probably due to the metabolic effects of damage to tissue rather than caused directly by thrombosis itself.

The different properties of arterial and venous blood have been examined and it has been shown that there is a higher activator concentration in blood from a vein though other components of the system have similar levels irrespective of whether the blood is venous or arterial.

In obese subjects the concentration of activator is reduced but there does not appear to be any increase in the levels of anti-plasmin or anti-activator. When, however, obese patients are starved under controlled conditions with the primary objective of reducing their weights, a peak of fibrinolytic activity appears in the initial stages of starvation.

When the drug atromid was administered to patients it lowered plasma fibrinogen and serum cholesterol. A transient rise in plasma plasminogen was also recorded and these studies illustrate how examination of the different components of the blood coagulation and clot dissolution systems can aid in unravelling the mechanism of the action of drugs of this nature.

#### Platelet adhesiveness

This is probably an important factor in the formation of blood clots since one of the earliest events in the normal clotting sequence is the accretion of platelets in large numbers.

The adhesiveness of these small cells has been studied using two methods, *in vivo* and the other *in vitro*.

Dietary factors have been studied for their effects and it has been shown that ingestion of one dose of glucose or sucrose or the prolonged administration of excessive dietary sucrose over several weeks shows little effect. Likewise no change has been observed following fatty meals.

Cigarette smoking has been shown usually to increase platelet adhesiveness. It is known that there is a predisposition to thrombosis in patients who have undergone surgical operations and studies on such patients have shown marked changes in platelet adhesiveness which may have some relevance to early post-operative thrombosis.

Infusion of dextran has been shown to reduce platelet adhesiveness and the anti-thrombotic effects of this substance are being further studied.

#### Lipid clearing factor

This effect of clearing of lipid is observed when, after administration of heparin, plasma from the subject is incubated *in vitro* with a suitable fatty substrate. It is probably one of the mechanisms which results in the clearing of lipid from the blood after a meal.

Observations have been carried out on this parameter in subjects of different age and sex and it has been shown that in men over about 50 years of age there occurs a sharp fall in the amount of lipid clearing factor. Men under 50 have levels similar to those of women of all ages. It is not possible to distinguish a difference between atherosclerotic men and normal men though patients with hypertension show significantly higher than normal levels even in the absence of cardiac symptoms. In patients suffering from congestive cardiac failure the clearing factor level rises and falls only after complete recovery from the condition.

Inhibitors of clearing activity exist. These are increased in apparently normal men over 50 in parallel with the fall in clearing activity. There is less inhibition than normal in subjects with cardiac failure, inhibitors increasing as failure is treated. In hypertensive subjects, however, inhibitors are not reduced in association with the increased clearing activity, so that the mechan-

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isms of increased clearing in hypertension and in cardiac failure appear to be different.

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#### 7. Effects of smoking on the cardiovascular system

(Dr J. P. Shillingford, Dr D. W. Irving, Mr G. Makin, Dr B. Pentecost, Dr T. Yamamoto and Dr L. Zatz, Royal Postgraduate Medical School, London)

The Council has supported research into the effects of smoking on the cardiovascular system in health and disease carried out in association with the Cardiovascular Research Group of the Medical Research Council at the Royal Postgraduate Medical School of London, of which Dr J. P. Shillingford is Director. The programme of the Group is to carry out research into the effects of smoking on blood flow in normal subjects, in patients with coronary arterial disease, in hypertensive patients, in patients with valvular disease and in patients with peripheral vascular disease. The Group will also be examining the effect of smoking on cerebral blood flow.

A dye dilution technique developed by the Group for recording serial changes in cardiac output was used to study the effects of cigarette smoking and intravenous nicotine on the cardiac output. "Sham" smoking an unlit cigarette or smoking without inhaling caused only slight changes in the cardiac output of normal subjects. Smoking with inhaling and the injection of intravenous nicotine increased the rate of the heart, total cardiac output, stroke volume and the systolic blood pressure. The increase in the stroke output of the left ventricle was up to 100 per cent of its resting value.

Patients who had had myocardial infarction or were suffering from ischaemic

mic heart disease were studied by measuring the effect of smoking cigarettes and of injections of nicotine tartrate on their cardiac output, blood pressure and pulse rate. There followed the usual increase in heart rate and blood pressure but some patients among the post-myocardial infarction group showed a marked fall in stroke volume and cardiac output while smoking. The fall in stroke output was as high as 40 per cent of the resting output. It was possible to produce left-sided cardiac failure in some such patients. Dr Shillingford considered it reasonably clear that cigarette smoking was probably harmful to the majority of patients who had had a coronary thrombosis in that it threw an extra load on a heart which was already damaged by disease.

Studies have been made on the effect of nicotine in patients with valvular incompetence. In those with aortic or mitral incompetence, the effect of smoking or the administration of nicotine was to increase the peripheral resistance and the heart rate and produce more effective valvular incompetence. Although the stroke output of the left ventricle in mitral or aortic incompetence increased due to the greater resistance, the effective cardiac output fell. Dr Shillingford considered that in this condition also smoking was contraindicated.

Techniques have been developed for the measurement of venous flow and are being used for the study of peripheral vascular blood flow and muscle metabolism and for the study of renal vein flow. Preliminary studies have been carried out to investigate the reports that nicotine may have a direct action on peripheral blood vessels in addition to its effect in stimulating ganglia. Preliminary findings suggest that nicotine has a local dilating effect but more evidence is necessary.

The response of nine patients with atherosclerotic obstruction of lower limb vessels to intravenous nicotine injections has been studied. As well as an increase in cardiac output there were flow and resistance changes in the ischaemic limb. An initial increase in flow in the calf was noted for about three minutes, followed by a temporary constriction and decrease in flow for about the same period.

Work has also been carried out with dogs on certain reflexes originating in the heart. These reflexes may be excited chemically by nicotine, among other agents, though their physical stimulus is unknown. Activation of the reflex results in a slowing of the heart, fall in blood pressure, fall in power of contraction of the heart and an increase in muscle blood flow. A similar pattern has been seen in man following acute myocardial infarction, though it remains to be seen whether reflexes of this sort can be excited by the presence of a myocardial infarct.

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8. Factors related to vascular disease  
(Dr K. J. Kingsbury, St Mary's Hospital, London)

The Council contributed for a period of three years to the support of research, organised by Dr Kingsbury, into the relationship of different degrees and types of disease of the aorta, iliac and femoral arteries to biochemical abnormalities, particularly in blood lipids, and a number of personal and environmental factors. The hypothesis underlying the research project was that atheromatous vascular disease could not be considered homogeneous biochemically or biologically and that different forms or degrees of the disease might be associated with different biochemical abnormalities and different personal or environmental factors.

The first requirement was to develop an improved method of classifying arterial abnormality. A procedure was developed for classifying arterial disease on the basis of visual inspection of the arteriograms, measurement of the length of irregularities in arterial walls in the arteriograms, comparison of X-ray reports with arteriograms, and observations of surgeons at operations. The percentage involvement of the aorta, iliac and femoral arteries was calculated. This procedure was considered to be reasonably reproducible and was chosen as an acceptable beginning.

The arterial gradings were then compared with the results of biochemical tests. The biochemical determinations proposed included fatty acid composition of plasma esters, cholesterol, phospholipids, glycerides, unesterified fatty acids and uric acid levels of the plasma, response to glucagon, adrenaline and glucose injections, biochemical response to smoking and oral glucose tolerance. Glycolysis was used as an example of cell enzyme activity.

Information was also sought on personal factors including age, weight, blood pressure, blood group, electrocardiograms, health history, occupation, body measurements, place of residence, diet, smoking habits, exercise and exposure to mental stress.

A pilot study was carried out. The results supported the hypothesis that atheromatous vascular disease could not be considered homogeneous biochemically or biologically. Differences were found between peripheral and central disease, severe and mild disease, and patients with aneurysms. Glucose tolerance was found to vary with the type of disease. Patients with atheromatous vascular disease appeared to include more cigarette smokers than the population generally. It was observed that smoking could reduce the blood glucose level, even in patients with a reduced glucose tolerance. It was also found that, in several subjects, red cell glycolysis could be sharply increased in the hours after smoking ordinary or low nicotine cigarettes or small cigars, and that this effect survived in stored plasma. Injections of adrenaline did not produce these effects.

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### 9. Metabolism and the cardiovascular system

(Professor W. J. H. Butterfield, Dr N. Cohen, Dr M. Ilahi, Dr I. McGill, Dr T. Northfield, Dr Z. Rihan, Dr J. Wakelin, Guy's Hospital Medical School, London)

In the 1962 Bedford Diabetic Survey a large number of unrecognised diabetes cases were found, and workers in Professor Butterfield's Department uncovered a correlation between the height of the blood sugar during the glucose tolerance test and the prevalence of vascular disease. The possibility exists that these two findings may explain in part the rising incidence of coronary thrombosis. Increased understanding of the factors involved in obstruction to the blood flow in peripheral vessels should provide information not only about the pathology of such conditions as gangrene, but shed some light on events in more central and vital organs such as the heart and brain. The work which Professor Butterfield and his colleagues have been carrying out in this field, with support of a grant from the Council, falls into two main categories, namely, studies based on blood flow measurements and metabolic investigations. These workers have been concentrating more on the first aspect, as a means of categorising the cases for subsequent metabolic investigations. Two techniques have been developed to follow the blood flow in the peripheral vessels, one for the legs based on foot plethysmography and the other for the fingers based on flow calorimetry. These methods are now in wide and general use and were used in the investigation and treatment of about thirty patients in Guy's Hospital during 1965. In both types of case it is desired to estimate the degree of vascular disease and this requires that an estimate be made of the maximal flow which will pass through the blood vessels which in turn demands removal of all tone of the blood vessels.

In the case of the fingers this is achieved by body heating, and forty-seven cases of Raynaud's disease have been studied. A trial of the fibrinolytic biguanide, phenformin, in Raynaud's disease associated with rheumatoid arthritis is being organised.

The peak blood flow in the foot is induced by arterial occlusion for 20 minutes and measured plethysmographically. This technique has been used to seek the effects of various diseases and treatments on peripheral blood flow. It has been used to show that there is impaired blood supply to the feet in diabetics, and it is hoped it will provide a means for detecting the progress of disease and the effects of treatment. In a study covering thirteen cases of peripheral vascular disease with and without mild diabetes it was not found possible to trace any significant effect on peak blood flow for cyclospasmol, nor any change of vascular tone as judged by the resting blood flow. In a second study, the response of eleven patients to clofibrate (atromid) was investigated using the same method. The results were equivocal, some patients showing excellent clinical responses and improved peak blood flows, others showing no response.

These results indicated that additional information about hyperemia was required before classifying patients and treating them. This led to a study of the biochemical basis of reactive hyperemia and control of muscle blood flow. One question of interest was whether the biochemical mechanism of

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reactive hyperaemia might itself be affected in diabetes and atherosclerosis. This led to an investigation of the mechanism of reactive hyperaemia, the first results of which have been published (Abrams, Barker and Butterfield, 1965). They show that ischaemia makes the muscle arterioles extremely sensitive to the level of circulating catecholamines and the muscle vasodilatation after occlusion of the circulation may be due at least in part to this process, making the vessels sensitive to the vasodilatory effect of circulating adrenaline. It seems clear that this mechanism is affected by angiotensin. It has become apparent that it is necessary to measure blood viscosity in patients before attempting to test therapeutic agents such as cyclospasmol and clonibrate. This is now being undertaken together with studies of sympathetic tone and both have been shown to have considerable clinical value.

Other studies have been undertaken using an intra-arterial catheter which can be passed down the femoral artery to pierce and re-canalise the atherosclerotic blockage in the artery. The only difficulty is that, as the blood flow returns, a large number of emboli are carried down into the lower limb, so that foot blood flow may not be improved. It is planned, therefore, to investigate the possibility of collecting the fragments of arterial wall by a second puncture and suction below, or by developing a new catheter for suction from above.

Other developments include work to be carried out with an arterial endoscope which is at present being constructed. A study on the clinical application of hyperbaric oxygen in the treatment of ischaemic gangrene has also been begun recently.

In regard to metabolic studies, further epidemiological work on the correlation between hyperglycaemia and electrocardiographic changes in diabetes is being undertaken in a new study which has just been started.

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#### 10. Retinal microcirculation in vascular disease

(Dr C. T. Dollery, Royal Postgraduate Medical School, London)

The Council is contributing to the support of research by Dr Dollery into methods for studying blood circulation through the arteries and blood vessels of the retina of the eye under varying circumstances in man and animals. One of the first experiments carried out was to observe the effects of retinal arteriolar occlusion. Following this, in co-operation with the Eye Pathology Unit of the Institute of Ophthalmology, a detailed correlation of the

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changes in blood flow and retinal structure following embolisation has been made.

The retinal arterioles spread outwards from the optic disc like the branches of a tree and like the smaller vessels of the brain and heart they have no direct anastomoses between one another. It had been thought that blocking a small arteriole of this kind would lead to immediate cessation of flow through its territory, but the present work has shown clearly that this does not happen and has given a new insight into the mechanisms whereby larger diameter channels develop which carry blood flow from normal vessels into the territory of the one which has been blocked.

Fluorescence angiograms performed immediately after embolisation have shown that the main trunk of the arteriole is blocked at the site where the embolus lodges but the vessel fills from the periphery via capillary collaterals from the surrounding patent arterioles. This is possible because the capillary bed is an extended branching network rather like a sheet of wire netting and a pathway exists through the capillary bed along its high pressure end between one arteriole and the next. This phenomenon has been named "capillary collateral flow" and is important for two reasons. Firstly, it permits some flow to continue within the territory of a blood vessel that has been blocked. Secondly, dilatation of some of these capillary channels allows the blood flow to increase eventually to a point where flow is restored almost to normal. In the pig this sequence of flow changes takes four to six weeks, but some dilatation of capillary collaterals is evident after only three or four days.

Normal retinal blood vessels do not leak, but abnormal vessels in patients with high blood pressure and diabetes often do. The experiments in the pig have shed light on how this happens. Glass microspheres which lodge in arterioles often move slowly down to a more distal position and the length of arteriole which has been transversed in this way often leaks the fluorescence dye. A microscopic study of the lining of these small arterioles shows that leakage takes place when the endothelial cell has been damaged and haemorrhage sometimes occurs from these sites.

The ability to observe the sequence of changes in a blood vessel which is affected by a pathological process over hours, days or weeks is a substantial advance in the understanding of vascular pathology. The work is continuing.

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#### Section 4. Birth Weights and Perinatal Mortality

##### Smoking in relation to birth weights and perinatal mortality (Dr. N. R. Butler, National Birthday Trust Fund, London)

This investigation consisted of the analysis of data concerning the pregnancies and deliveries of 93 per cent of all births (approximately 17,000) occurring in England, Scotland and Wales during one week in March, 1958, and 94 per cent of all stillbirths and deaths of infants up to one month of age (approximately 7,000) occurring during the months of March, April and May, 1958. The basic data were obtained from a detailed questionnaire completed at the birth of the infant.

The aim of the investigation was to relate the outcome of pregnancy, as far as possible, to factors thought to affect the survival, health or birth weight of the baby. Smoking during pregnancy had been reported to be associated with low birth weight, although there have been conflicting reports about its association with foetal or infant death, in part because of the small numbers in previous surveys.

Preliminary examination of the data revealed that about a third of the mothers had smoked during the second half of pregnancy. This period was chosen because it was thought to be the time during which smoking might exert a maximum effect on the baby. The proportion of babies weighing 5½ lb. or less (the international definition of prematurity) was found to be significantly greater in smokers than non-smokers, and to increase with the number of cigarettes smoked. Although there was an associated excess of abnormally short pregnancies, this factor did not entirely account for the reduction in average birth weight of the infants of smoking mothers. Both the stillbirth and the neonatal death rates were significantly raised in the smoking group.

However, as in previous findings, there was a marked excess of mothers of low social class, high parity (number of previous babies) and advanced maternal age among the smokers. In those groups, Butler and Bonham (1963) found perinatal mortality to be higher than average. These factors are associated one with another and mothers of lower social class more often have larger numbers of children and at later ages than those of higher social class. Furthermore, abnormally early delivery and particularly low birth weight are more common among such mothers. It was clear that only a sophisticated statistical approach could disentangle these effects and answer the question whether smoking during pregnancy was associated with low birth weight and increased infant and foetal death regardless of any other adverse factors.

This analysis was first carried out using multiple regression analysis (Feldstein, 1965) of the proportion of deaths and low birth weight babies, with variables such as social class, age, parity and length of pregnancy. From these results it appeared that smoking was associated with low birth weight and increased stillbirth and neonatal death rates, even when these other factors were discounted. To test the validity of these results, a different method of analysis, involving a logit transformation of proportions is being

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carried out. Most of this work has now been completed and confirms the results obtained by Feldstein's methods.

Other questions that have been raised in the past are on the distribution of blood groups in smokers and non-smokers, the incidence of toxæmia in pregnancy in smokers and non-smokers and whether there is an alteration in sex ratio in the babies of smokers. The findings from the present series were that there was no difference in blood group distribution between mothers who were known to have smoked, and those who did not. As one would expect in a group of mothers of higher mean parity, pre-eclamptic toxæmia was in fact less common amongst smokers than non-smokers. The sex distribution of infants born to mothers who smoked did not differ from that found in babies of mothers who did not smoke.

The Council contributed to the cost of the work described.

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- Butler, N. R. and Bonham, D. G. (1963). *Perinatal mortality*. Edinburgh, E. & S. Livingstone, Ltd.  
 Feldstein, M. S. (1965). *Br. J. prev. soc. Med.*, 19, 135.  
 Feldstein, M. S. and Butler, N. R. (1965). *Br. J. prev. soc. Med.*, 19, 123.

#### Section 5. Main Causes of Death

Differences in mortality patterns of South African born men and women, British and other immigrants to South Africa, and residents of England and Wales  
 (Dr G. Dean)

The Council supported a comparative study, which has been completed, by Dr G. Dean of all causes of death of South African born men and women, British and other immigrants to South Africa, and residents of England and Wales. The purpose of the inquiry was to find out whether there were significant differences between these population groups in any of the main causes of death, since these might point to possible effects of differences in genetic factors or in environmental factors in South Africa or the country of birth.

Age-standardised comparisons of a number of the most important diseases showed marked differences between the white South African born population, immigrants from the U.K. and residents of England and Wales. Male lung cancer was highest in England and Wales, intermediate in British immigrants to South Africa and lowest in white South African born men. The male mortality rate from bronchitis in England and Wales was 1.4 times the rate of white South African born men. Dr Dean reported that although white South African men smoked more than men in England and Wales, their risk of dying from bronchitis was much less, while the British immigrants who settled in South Africa lost the high British risk of dying from

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chronic bronchitis. Coronary thrombosis was a major cause of death among white South Africans and was much higher than the corresponding rate in England and Wales. Immigrants from Britain had a coronary thrombosis mortality rate only a little higher than the population of England and Wales at ages 45 to 69, although most of the immigrants settled in the cities where the South African born population had a higher risk than the average for South Africa as a whole.

Dr Dean added that it was becoming increasingly possible to identify high risk groups for different causes of death and that people in these groups could often be advised how they should alter their way of life and so lessen their risk.

*Reference*

Dean, G. (1965). *S. Afr. med. J.*, 51 July, Supplement.

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Part IV. PSYCHOLOGICAL ASPECTS OF  
SMOKING

## 1. Some correlates of smoking

(Dr F. E. Emery, Tavistock Institute of Human Relations)

In 1964, the Council agreed to participate in a study by Dr F. E. Emery of the Tavistock Institute of Human Relations, carried out in co-operation with Public Attitude Surveys Ltd, which included research into the motives for smoking. The study was designed and its analysis has proceeded on the hypothesis that tobacco was one of a class of objects that are commonly used to regulate and control the emotions, either because these emotions are felt to be painful in themselves or because, whether painful or pleasant, they intrude on some ongoing commitment.

A probability sample of 2,511 English adults was interviewed. A comprehensive questionnaire was used which included the Tomkins-Horn picture arrangement tests, the Witkin embedded figures test, the short form of the Maudsley Personality Inventory and a short vocabulary test. Dr Emery has not yet reached the stage in the analyses of his data when systematic conclusions are possible but he has added to knowledge of factors which are correlated with smoking.

Some personality types are more prone than others to be smokers rather than non-smokers, cigarette rather than pipe or cigar smokers, heavy rather than light smokers, inhalers rather than non-inhalers. Professor H. J. Eysenck (1960) had found an association between cigarette smoking and extraversion (as measured by the Maudsley Personality Inventory). This has been confirmed by Dr Emery. Professor Eysenck failed to find an association between cigarette smoking and neuroticism, and Dr Emery's findings suggested that there might even be a negative relation between the two. Dr Emery considered, however, that the joint use of the extraversion/introversion and the neuroticism (or emotional stability/instability) scales of the Maudsley Personality Inventory did not adequately define basic personality types. He therefore subdivided the extraverts and introverts into the four categories:

- A. Extraverts preoccupied with other people (i.e. social extraverts)
- B. Extraverts preoccupied with action on the outside world (i.e. object extraverts)
- C. Introverts preoccupied with inner emotional processes
- D. Introverts preoccupied with conceptual thinking

According to Dr Emery, A and D were basically the more stable modes of life. Dr Emery has reported that preliminary calculations showed that these personality types showed some relations with occupational type and the

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presence of an intuitive or logical mode of thought. These factors in turn appeared to affect the influence of personality type on smoking.

After analysing the situations most associated with the use of tobacco, Dr Emery concluded that his data implied a theory in which the three main causes of smoking, in order of importance, were a need to concentrate, a need to offset boredom and a need to relieve tension. More generally, smoking appeared to raise the threshold to conscious feelings of distress without any direct effect on excitement or other effects.

A full report will be published in due course.

#### References

Eysenck, H. J., Tarrant, M., Woolf, M. and England, L. (1969). *Br. med. J.*, 1, 1413.

#### 2. Behavioural effect of nicotine absorbed during smoking (Professor H. J. Eysenck, Institute of Psychiatry, University of London)

The Council agreed in 1961 to support, as a follow-up to studies that Professor H. J. Eysenck had carried out into the effects of stimulant drugs on behaviour and personality, a study of the behavioural effects of nicotine absorbed during smoking. While Professor Eysenck did not suggest that nicotine was the sole reason for smoking, he considered that nicotine might have a stimulant effect that was a prime factor in the continuation of the smoking habit once it had developed. Professor Eysenck therefore designed a series of experiments to examine the psychological effects of nicotine, to discover whether small doses of nicotine, equivalent to those absorbed by smoking, would produce stimulant effects, and also to give some insight into the manner in which this stimulation occurred. The tests were chosen to cover a wide range of behavioural functions from the effects of nicotine on sensory responses to its effect on complex motor skills.

A first series of psychological experiments led Professor Eysenck to the conclusion that nicotine-induced changes in the performance of these experiments were consistent with the hypothesis that nicotine acted as a stimulant drug, and that the behavioural effects produced by it were similar to those produced by other stimulants. The pattern of changes caused by nicotine was confounded to some extent by two other factors, namely, sex differences and differences between smokers and non-smokers in personality and tolerance to nicotine.

Further series of experiments were carried out using both human subjects and the Maudsley reactive and non-reactive strains of rats. Some of the experiments with human subjects were designed to see whether introverts had lower sensory thresholds than extraverts, whether nicotine lowered sensory thresholds, and whether there was any interaction between these two variables. In some though not in all the experiments, the results confirmed the hypotheses advanced.

The experiments with rats showed that injections of nicotine tartrate improved the efficiency of learning. Some further experiments with human subjects followed. Professor Eysenck has put forward the suggestion that

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habit of smoking a cigarette during a rest interval when engaged on some mental task may be useful in that the nicotine consumed may aid "consolidation", in which several associations become fixed or consolidated, and thus enhance learning and memory.

*Reference*

Warwick, K. M. and Eysenck, H. J. (1963). *Life Sciences*, 4, 219.

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## Part V. PHARMACOLOGICAL ASPECTS OF SMOKING

### *Section 1. Pharmacological Research at TRC Laboratories, Harrogate*

Three main lines of pharmacological research are being carried out at TRC Laboratories, Harrogate. These are investigations of the effects of smoking and of nicotine on the central nervous system, on the peripheral nervous system and on animal behaviour. Some experiments have also been carried out on the effects of nicotine on the mobilisation of glucose and free fatty acids and on the deposition of body fat.

#### *Some effects of nicotine on the central nervous system of cats*

In order to study the direct effects of nicotine on the central nervous system, uncomplicated by any peripheral actions of nicotine, experiments were carried out in which nicotine was injected directly into the cerebral ventricles of cats, using the peristone technique devised by Professor W. Feldberg at the National Institute for Medical Research. One of the characteristic effects of nicotine injected by this route in amounts ranging from 1-100µg (base) was vigorous twitching of the ears. This unique response was first reported by Armitage, Milton and Morrison (1965) and by Hall and Reit (1965). The site of action of nicotine in eliciting this response has been localised to the upper cervical cord. This action of nicotine could be potentiated by physostigmine and neostigmine under suitable conditions, probably due to inhibition of brain cholinesterase. A likely mechanism by which nicotine caused twitching of the ears would appear to be mimicry of the action, or perhaps release, of acetylcholine in the central nervous system (Armitage, Milton and Morrison, 1966; Armitage, Hall, Milton and Morrison, 1966). The first results of further work which is still in progress are tending to confirm that one action of nicotine in the cat is to release acetylcholine in the central nervous system.

#### *Effects of nicotine and tobacco smoke on blood pressure and release of catecholamines from the adrenal glands*

Cigarette smoke was introduced into the lungs of a cat under carefully controlled conditions and the resulting rise in blood pressure compared with the rise caused by nicotine acid tartrate injected intravenously (Armitage, 1966). The effect on blood pressure of a single puff of cigarette smoke was almost identical with the effect of half the amount of nicotine in the puff injected intravenously. The last puff of a cigarette was found to contain two to three times as much nicotine as the first puff. It is likely that the nicotine intake during the smoking of a cigarette is equivalent to a series of small but pro-

gressively increasing intravenous injections. The rise of blood pressure caused by tobacco smoke appeared to be due to a substance or substances with the properties of nicotine, though it is not possible to state that the increase in blood pressure was due solely to the nicotine content of the smoke. It seems likely that only smokers who inhale deeply and rather frequently will attain a sufficiently high blood nicotine level to cause a release of catecholamines from the adrenal glands. Whether the amounts released have any physiological significance has not been elucidated.

The rise in blood pressure resulting from a small intravenous dose of nicotine in the cat did not appear to be entirely accounted for by stimulation of sympathetic ganglia, release of catecholamines from the adrenal glands or by chemoreceptor stimulation. The possibility remained that direct stimulation of the vasomotor centre was involved.

#### *Effects of nicotine on spontaneous motor activity of mice*

The motor activity of mice was measured in automatically recording activity boxes. The effect of nicotine on exploratory behaviour and spontaneous activity was tested in three strains of mice, singly and in pairs, in an extensive series of experiments. The nicotine was usually given subcutaneously (0.1–0.3 mg/kg), and in a few experiments nicotine was injected directly into the tail vein through a polythene cannula (0.0005–0.002 mg/kg/30 sec.) for 10–15 minutes. In all these experiments there was a dose-dependent reduction in motor activity though there were usually a few mice that were unaffected or even the highest dose (Morrison and Armitage, 1966). Different strains of mice varied in their sensitivity to nicotine. Whether the reduction in activity seen with the smallest doses of nicotine was a behavioural effect of nicotine, or whether it was a toxic manifestation, was not certain. Reduction in motor activity following relatively high doses of nicotine also occurred in rats.

#### *Effects of nicotine on free-operant behaviour of rats*

The effects of four dose levels of nicotine (0.05–0.4 mg/kg) injected subcutaneously were studied in hooded rats trained to press a lever on three different schedules of reward (Morrison and Armitage, 1966). In one experiment, the rats had to press the lever fifty times for each reward, in another they were rewarded on the average once every two minutes, and in the third experiment the rats were rewarded only when there was a pause of 20 seconds or more between responses. The effects of nicotine were compared with those of the stimulant drug amphetamine.

On the count schedule of reward, nicotine acted as a stimulant, usually after an initial period of depression, but the results were irregular due to the small numbers of animals. On the variable interval schedule, the two highest doses caused first a reduction and then an increase in the rate of lever pressing; the two lower doses did not cause initial depression and the stimulation was less. This is an important observation showing that the effect of nicotine on behaviour can be totally different if behaviour is measured shortly after subcutaneous injection or 30 minutes later. The smallest dose of nicotine (0.05 mg/kg) which resulted in an increase in the rate of lever pressing is

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considered to be roughly equivalent to a "smoking" dose. The highest dose of 0.4 mg/kg, on the other hand, which resulted in reduction of lever pressing before stimulation, may be a mildly toxic one. Nicotine had less effect on rats in the experiments in which the rats were rewarded only when they pressed the lever relatively slowly.

Both nicotine and amphetamine acted as stimulants on all three schedules of reward. Nicotine had a greater effect than amphetamine on the variable interval schedule, but amphetamine had the greater effect on the other two schedules.

*Effect of nicotine on blood glucose and free fatty acid levels in the cat*

Effects of intravenous injections of nicotine on blood glucose and free fatty acid levels have been estimated in the anaesthetised cat (Milton, 1966). Ten injections of a low dose of nicotine given during 10 minutes elevated both glucose and free fatty acid levels. This effect was entirely due to stimulation of the adrenal gland. Larger amounts of nicotine caused a large increase in the blood glucose level, but the effect on free fatty acids was transient. After removal of the adrenal glands, the effects on glucose were much reduced, whereas elevation of free fatty acids occurred. These observations showed that mobilisation of free fatty acids is depressed during hyperglycaemia.

*Effects of chronic administration of nicotine to rats*

A group of rats was given subcutaneous injections of nicotine (0.5 mg/kg) twice a day for six months to see whether an effect of nicotine on body weight could be demonstrated. There were no significant differences between the two groups in body weight, heart weight, kidney weight, plasma cholesterol levels (in contrast to observations by other workers) and adrenal gland adrenaline and noradrenaline (total content). The amount of total body fat in the control animals was 57.2 gms or 144.5 gms per kg body weight, which differed significantly from 37.0 gms or 100.6 gms per kg body weight of the nicotine treated rats. The nicotine treated animals had significantly lower liver weight per kg of body weight, lower blood glucose levels and significantly higher weight of adrenal glands per kg of body weight. Since the liver is a storage organ for carbohydrate, the lower liver weights and lower blood glucose levels suggested that prolonged nicotine administration had depleted the carbohydrate reserves.

*References*

- Feldberg, W. (1963) *A pharmacological approach to the brain from its inner and outer surface*.  
Edward Arnold, London.  
Other references will be found in the list of papers by the scientific staff of the Tobacco Research Council Laboratories (p. 92).

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## Section 2. Pharmacological Research by Independent Scientists

### 1. Pharmacological actions of nicotine and related alkaloids from tobacco smoke on the central nervous system (Dr M. S. G. Clark, School of Pharmacy, University of London)

Under the supervision of Dr M. J. Rand, Dr Clark studied in isolated tissues, animal and man, the pharmacological actions of nicotine and related alkaloids which had been identified in cigarette and tobacco smoke. The alkaloids tested were nicotine, nornicotine, metanictine, anabasine, myosmine, nicotinic, 2:3-dipyridyl, 3-methyl-6-(3-pyridyl)-tetrahydro-1:2 oxazine, dehydrometanictine, N-methyl anabasine, cotinine, nornicotyrine, pyridylmethyl ketone and pyridyl propyl ketone. The experiments showed that nicotine and the other tobacco smoke alkaloids possessed a depolarising action at the junction of nerve and voluntary muscle and caused neuromuscular blockade. The parallel dose/response curves and the similar shape and characteristics of the effects of the nicotine-like alkaloids on two isolated tissues suggested a similar site of action for all of them in these preparations. The alkaloids which were active in producing neuromuscular blockade blocked the monosynaptic patellar tendon reflex in small doses, while large doses inhibited the polysynaptic flexor reflex. Dr Clark suggested that the effects were due to stimulation of inhibitory mechanisms rather than blockade of excitatory neurones. There was a peripheral component in this action of nicotine which was not evident with the other alkaloids.

The effects of cigarette smoke, of a nicotine aerosol and of a nicotine aerosol mixture, administered via a tracheal cannula, on the knee-jerk of the anaesthetised cat were also investigated. Administration of cigarette smoke and nebulised nicotine produced effects which were indistinguishable from intravenously injected nicotine, and there was the same rise or fall of blood pressure for an equivalent blockade of the knee-jerk. The similarity between the effects on blood pressure and the effects on the knee-jerk of nicotine and cigarette smoke suggested that the effect of cigarette smoke was due to the nicotine it contained.

The evidence also suggested that nicotine, which occurred in tobacco smoke in considerably higher concentrations than the other alkaloids, was the most potent alkaloid occurring naturally in tobacco smoke. Dr Clark estimated that the maximum contribution by the alkaloids other than nicotine in tobacco smoke to the pharmacological activity of the smoke could not exceed 0.01 per cent of the contribution by nicotine.

In human subjects, inhalation of the smoke from one cigarette blocked the knee-jerk in light smokers but was without effect in heavy smokers. The effect on light smokers was dependent on the nicotine present in the smoke. Smoke from cigarettes, specially made to contain only 0.13 per cent nicotine, instead of the usual 2.1 per cent, was described by the subjects as "insipid" and had no effect on the reflex responses. The suggestion was made that knee-jerks were most easily blocked by cigarette smoke in people who reacted most readily to specific stress. It was also suggested that the suppression of

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spinal reflexes involved in continual irrelevant muscle twitches contributed to tranquillisation, and that this could explain the tranquillising effect attributed to tobacco smoke.

A study of two aspects of the activity of nicotine on the central nervous system in rats led to the suggestion, which was consistent with the view of a number of other research workers, that some actions of nicotine on the central nervous system were mediated through or associated with acetylcholine.

It was also shown that the acquisition of positive reinforcement conditioning by rats, i.e. the pressing of a lever for a water reward, could be potentiated by nicotine (0.4 mg/kg subcutaneously). A smaller dose (0.1 mg/kg) did not produce this effect. Potentiation of acquisition in similar situations has been shown by other workers. The effect might be due to an increase of exploratory activity of the animals on being placed in the conditioning apparatus. This explanation has been used to explain a similar effect with drug mixtures which are used clinically to relieve anxiety.

In order to study the absorption of nicotine by the smoker from tobacco smoke, measurements were made of the nicotine in the blood leaving the lungs of anaesthetised dogs before and after the introduction of tobacco smoke. The results suggested that the nicotine that was found in the blood must have been transferred into the blood stream almost immediately. Nicotine absorbed from cigarette smoke was distributed in the blood in essentially the same way as nicotine administered by a series of small injections.

The general conclusion of Dr Clark was that there was much evidence to support the assumption that smokers smoked in order to obtain the pharmacological effects of the nicotine in the tobacco. Dr Clark suggested that a reason why nicotine may cause an overall increase in general well-being of a person might be due to the level of motorneurone activity and of muscle tone being higher in some people than in others. These levels of activity and tone were especially liable to increase in a stressful situation. Nicotine absorbed during smoking may reduce motorneurone activity by stimulating an inhibitory system in the spinal cord (the Renshaw cell). In addition there may be some inhibition of motorneurone activity arising from the effects of nicotine at higher levels. More than one mechanism may be involved, each being stimulated by nicotine. The final effect would be a composite one depending on route, method of administration and dosage.

#### References

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Clark, M. S. G., Rand, M. J., and Vanov, S. (1965). *Arch. int. Pharmacodyn.*, 156, 363.

## 2. Some pharmacological actions of nicotine on the peripheral autonomic nervous system (Dr S. Vanov, School of Pharmacy, University of London)

The actions of nicotine on the peripheral autonomic nervous system were studied indirectly by carrying out a pharmacological analysis of the mechani-

cal responses of several smooth-muscle neuro-effectors to nicotine and to other ganglion-stimulants.

#### *Parasympathomimetic effects*

These included contractions of the guinea pig isolated ileum and the rat urinary bladder *in situ*. It has been confirmed that the contractions of the guinea pig ileum are due to excitation of cholinergic neurons in the wall of the ileum. Out of 14 cigarette smoke alkaloids, tested on the guinea pig ileum, nicotine was the most potent. Nicotine had no appreciable effect on the rat isolated bladder, but injected intravenously in pithed rats it produced marked contractions of the bladder. Since these contractions were abolished by ganglion-blocking drugs, but not affected by adrenergic neuron blocking drugs, it was concluded that they resulted from excitation of cholinergic neurons the cell bodies of which are remote from the bladder wall.

#### *Sympathomimetic effects*

These included contraction of the dog isolated spleen, relaxation of isolated segments of intestinal muscle (rabbit colon, rat duodenum and guinea pig taenia caeci), relaxation of the guinea pig tracheal chain and stimulation of the rabbit or guinea pig isolated heart. Pharmacological evidence from experiments with various blocking drugs suggested that nicotine activates an adrenergic mechanism resulting in catecholamine-release. In experiments on the perfused dog spleen and on the rabbit or guinea pig heart, it was shown that the sympathomimetic effects of nicotine are associated with the appearance in the perfusate of a noradrenaline-like substance. In an attempt to characterize histologically the structures on which nicotine may act to produce its sympathomimetic effects, the "chromaffin" reaction and the histo-fluorescence technique for catecholamines were employed. No chromaffin cells were detected in the rat duodenum, rabbit ileo-colic sphincter and colon, or in the guinea pig trachea, heart and taenia caeci. All these tissues, except the trachea, contained a fair amount of fluorescing catecholamine-containing structures which were shown to consist predominantly of nerve fibres. It is suggested that nicotine acts on receptors in the adrenergic nerve terminals.

#### *Effect on the blood pressure of anesthetized cats or rats*

The rise in blood pressure upon intravenous administration of nicotine was found to result from stimulation of sympathetic ganglia and nerves and liberation of catecholamines from the adrenal medulla. The sympathetic structures appeared to be more sensitive to the circulating nicotine than the adrenal medulla.

#### *Effects of chronic administration of nicotine on the sympathetic nervous system*

In one series of experiments rats were exposed daily to cigarette smoke for 15 days. In another series, rats were injected daily with nicotine for 36 days. The rats of the first series did not differ from untreated controls in their reactivity to sympathetic activation elicited by drugs or nerve stimulation. The tissue catecholamine content of the rats of the second series was not significantly different from that of untreated controls. The results suggest

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that chronic repeated administration of nicotine does not lead to functional alterations of the sympathetic nervous system.

#### References

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Hollands, B. C. S., and Vanov, S. (1965). *Br. J. Pharmacol. Chemother.*, **21**, 327.  
Hukovic, S., Rand, M. J., and Vanov, S. (1965). *Br. J. Pharmacol. Chemother.*, **21**, 173.  
Vanov, S. (1965). *Br. J. Pharmacol. Chemother.*, **21**, 591.

### 3. Relationship between chemical structure and nicotine-like activity

(Dr R. B. Barlow, University of Edinburgh)

The purpose of this research project is to try to find out which chemical groups in nicotine give it its biological properties. Relationships between chemical structure and nicotine-like activity at sites in the peripheral nervous system had been studied by Barlow and Hamilton (1962) but, because compounds like nicotine are agonists and thus activate receptors, it is not possible to decide whether increase in activity indicates increases in affinity for receptors or increases in ability to activate them. In the present work the aim is to prepare series of compounds which are related to nicotine but which block receptors. These should indicate which chemical groups are associated with affinity for receptors and by comparing the results with the activity of the series of agonists it should be possible to infer which groups are associated with ability to activate receptors.

An accurate method has been developed for measuring the affinity constants of antagonists at the nicotine-sensitive acetylcholine receptors of the frog rectus muscle. This method is similar in principle to that described for the nicotine-sensitive acetylcholine receptors of the guinea pig ileum by Barlow, Scott and Stephenson (1965). However, because the responses with the frog rectus are so much slower in onset and take so much longer to wash out, the experiments have to be performed with automatic equipment over 24 hours, instead of being complete in 1 to 3 hours. Experimental methods have been devised to check that antagonists are competitive in that they are really combining with nicotinic receptors in such a way that the estimate of the affinity constant should be an absolute measure of affinity. Methods have also been evolved for measuring the affinity constants of partial agonists in this preparation (compounds which have some stimulant activity but cannot cause the tissue to contract maximally).

It has been shown that when the methyl groups in tetramethylammonium, dimethyl pyropidinium and dimethyl piperidinium salts are replaced by ethyl groups there is a loss in activity. As more groups are replaced, compounds are produced which are partial agonists. Ultimately antagonists are obtained. Measurements of the affinity constants of these latter indicate that replacement of methyl by ethyl leads to increased affinity. This suggests that the methyl group is important primarily for ability to activate receptors.

Similarly with the more potent ion, beta-pyridylmethyl trimethylammonium, the replacement of methyl by ethyl leads to a fall in nicotine-like

in

activity. On the frog rectus the methyldiethylammonium compound is a partial agonist and the triethyl compound an antagonist: the replacement of the last methyl group by ethyl increases affinity whereas it destroys the ability of the compound to activate receptors at all.

The effect of the position of attachment of the basic groups to the pyridine ring on affinity has been assessed by studying diethyl, di-*n*-propyl, and some di-*n*-butyl, aminomethyl- and aminoethyl-pyridines. These are antagonists or partial agonists and the compounds with the highest affinities are the alpha and gamma substituted derivatives with large substituents in the amino group. Barlow and Hamilton (1962) however found that the most active nicotine-like compounds are beta substituted derivatives (like nicotine itself) with small substituents in the amino group such as trimethylammonium. The ability of these compounds to become bound at the receptors therefore is not the only, or most important, property which gives them high biological activity. The beta pyridyl group and the small cationic group somehow endow these molecules with ability to activate receptors. It is hoped that further work may indicate why this should be so.

#### References

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#### 4. Nicotine absorption and metabolism in man

(Professor A. H. Beckett, Chelsea College of Science and Technology, London)

Data on the absorption and metabolism of nicotine in human beings are relatively limited and, using human volunteers, Professor Beckett is studying various aspects of this subject. It is proposed to determine blood concentrations of nicotine after intravenous injection and after absorption of volatilized nicotine by mouth and lungs. A detailed examination will be carried out on the excretion patterns of nicotine after smoking and a comparison made between non-smokers and those who smoke moderately and heavily. In this way, it is hoped to discover if habituation to nicotine leads to any alterations in the degree of its metabolism. Other points in these studies will involve detailed comparisons of the absorption of nicotine from different types of tobacco smoke, studies concerned with mouth absorption of nicotine, comparison between analytical techniques in the estimation of total nicotine per tobacco product, and it is hoped in time to set up computer models for the distribution, metabolism, excretion and re-absorption kinetics of nicotine in man.

#### 5. The effects of nicotine-like and muscarine-like compounds on the central nervous system

(Dr. E. Marley, Institute of Psychiatry, University of London)

The Council has agreed to support a study in which the effect of muscarine-like and nicotine-like substances on the central nervous system will be

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examined using chickens as experimental animals. Thus the effect of the muscarine-like substances should be specifically antagonized by hyoscine, whereas the nicotine-like substances should be antagonized by ganglion blocking agents like hexamethonium, mecamylamine, and pempidine. Studies will be carried out to determine whether, as on ganglia, nicotine has an initial excitant and then depressant activity and whether, in large or repeated doses, nicotine abolishes the effects of subsequent administration.

Work is also proposed on the localization of the site of action of these drugs which may act through the brain stem reticular formation. This could be verified by showing that the effects are obtained in chickens with a transection of the brain stem at the junction of the spinal cord and medullary bulb, but not with a transection of the upper mesencephalon. If the effects are still obtained after mesencephalic transection, then they must be mediated through systems located either in the cerebrum or throughout the brain. Dr Marley has already employed this type of experimental approach for localizing the site of action of the sympathomimetic amines.

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## Part VI. GENERAL MEDICAL RESEARCH

### *Immunology and Cancer*

#### 1. The thymus, immunity and tumours

(Dr. J. F. A. P. Miller, Institute of Cancer Research, London)

In recent years, there has been a considerable revival of interest in the thymus, particularly in its relationship to the development of immunological capacity. New knowledge of thymus physiology has been gained and has brought us closer to an understanding of immunological deficiency and autoimmune diseases.

The thymus is a relatively large organ in the infant, reaching its maximum size about the time of puberty after which it regresses. In late adult life, it is little more than a vestigial structure, but in the young adult it is still of considerable size.

The thymus forms part of the lymphoid system of the mammal. Available evidence favours the view that the thymus may be an important source of lymphocytes which circulate in blood and lymph and which may be distributed to the lymphoid organs.

Although immune processes have for long been known to be a function of the lymphoid complex, it is only recently that direct, unequivocal evidence has been obtained to demonstrate that the small lymphocyte is an immunologically competent cell, that is a cell which is fully qualified to initiate an immunological reaction when given the appropriate stimulus.

The thymus, through which the competent small lymphocytes do not normally recirculate in adult life, does not react to antigen by producing plasma cells and few, if any, of its small lymphocytes are competent to initiate transplantation immune reactions when appropriately stimulated. In spite of this inertia in taking part in immune responses, the role of the thymus in the development and maintenance of an adequate pool of competent cells is of fundamental importance as experiments performed since 1961 have shown.

A group of thymectomised mice was sacrificed before six weeks of age to examine their lymphoid tissues and blood. It was evident that the population of small lymphocytes throughout the body was severely reduced. The ability of thymectomised mice to perform immune reactions was tested and found to be deficient. For instance, foreign skin transplanted to such mice was not rejected but grew luxuriant tufts of hair and was tolerated until death of the animal. They had an impaired capacity to produce antibodies in response to some antigens such as sheep red cells.

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The earlier in life thymectomy was performed, the greater were the defects in immunological performance. It was then shown that thymectomy in the adult was eventually associated with defects in immune capacity when mice were challenged with a new antigen from six to nine months after thymectomy. The conclusion was made that the thymus throughout life ensures the maintenance of an adequate pool of immunologically competent cells and only when the pool becomes depleted as a result of the finite life span of its cells can defects in immune capacity become evident in the animal thymectomized in adult life.

An injection of spleen or lymph node cells taken from adult mice of the same inbred strain corrected the immunological inadequacies of neonatally thymectomized mice. It was thus probable that one of the defects of neonatally thymectomized mice was a reduction in the number of such cells. In fact, when the thoracic ducts of mice thymectomized at birth were cannulated, the cell output was 0.5 per cent to 1 per cent lower than that of normal control mice.

Implanting thymus tissue in a subcutaneous site or under the kidney capsule also corrected the defects of thymectomized mice. This suggested that the thymus implant could act not only by directly providing cells to the host but also by inducing the differentiation of host lymphoid precursor cells to fully competent small lymphocytes. It was concluded that the thymus influenced the development of the immune system by means of some humoral mechanisms.

Tumours were induced more readily in mice thymectomized at birth than in normal mice. The incidence and spectrum of tumour types was increased and the latent period decreased in the thymectomized mice.

This new work on the role of the thymus in the development of the immune system has focused attention on human diseases in which immunological abnormalities have been associated with thymus lesions. There are clearly immunological deficiency syndromes in infants which can be linked to a failure of thymus development. In conditions believed to be autoimmune in nature, when the body produces antibodies against itself, there are often associated thymus lesions and, sometimes, tumours. The exact relationship between thymus dysfunction and the development of such diseases is still unknown.

Many tumours induced by viruses and chemical agents are now known to have an antigenic individuality of their own, that is to have antigens "foreign" to those present in the host. Many experiments which have been performed indicate that neonatal thymectomy renders animals more susceptible to tumour inducing viruses and chemicals. Since these reactions are thymus-dependent it can be assumed that the thymus forms part of a "surveillance" system the function of which is to eliminate antigenically distinct cells before they can multiply.

The influence of the thymus on the natural resistance to tumours has opened up a new chapter in cancer research. Logical development of this field must increase understanding not only of the normal functions of the thymus but also of the role of immune processes in the resistance against induced (and possibly spontaneous) cancer. It will certainly shed some light

on the means by which resistance to cancer may be increased. It may even pave the way for eventual cancer prophylaxis.

The Council contributed to the cost of Dr Miller's research for a period of five years.

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## 2. Regression of chemically induced tumours and immunological mechanisms

(Presented by Sir Alexander Haddow and Dr P. Alexander, Institute of Cancer Research, London)

There is little doubt that the immunological approach to the cancer problem is among the most logical, but it is beset with many difficulties. The work of the Chester Beatty Research Institute over the past 15 years in the field of chemical carcinogenesis has indicated the likely importance of biochemical and functional losses and deletions, probably of systems essential to normal growth regulation in the origin of the cancer cell. If the problem is considered along these lines, it seems probable that the process involves antigenic differentiation and simplification leading to a cell which is defective in the normal capacity to evoke recognition by host system and consequent growth restraint. Thus, there seemed little or no evidence that cancer cells can stimulate an antagonistic response on the part of the body as a whole.

Lately, the position has been modified slightly due, in part, to a general revival of immunology in this field and also because of certain experimental observations which suggest a re-assignment of previous ideas.

Sir Alexander Haddow and his colleagues have carried out experiments in which part of a chemically induced rat sarcoma was removed surgically, the material made into a fortified antigen, and re-injected into the same animal to test for evidence of any reaction against the remaining primary tumour. The rat sarcoma system was used because it had been shown to be almost completely refractory to any kind of interference and did not regress spontaneously. These experiments were entirely negative except that, in three animals treated in this way, the primary sarcoma entirely disappeared. This, of course, is an isolated observation which has not yet been reproduced, but similar behaviour has been recorded recently in a small proportion of rats subjected only to an autograft from the primary tumour. A more consistent effect of autografting was observed when fibro-sarcomas were induced in rats by the implantation of pellets of benz(a)pyrene and then irradiated locally with X-rays, which resulted in delays in their growth. Enhancement of this delay could be demonstrated in about 50 per cent of the animals which received an injection of autochthonous tumour material previously rendered non-

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viable by irradiation *in vitro*. Sir Alexander Haddow and his colleagues are attempting to determine to what extent the immunogenicity of this tumour material might be enhanced, whether by X-radiation, alkylation, exposure to specific polysaccharides, antigenic fortification, etc.

The Council contributed to the cost of this research for a period of two years.

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## Part VII. BACKGROUND INFORMATION - RELATING TO SMOKING

### Section 1. Statistical

#### 1. Statistics of smoking in the U.K.

The publication of *Statistics of Smoking in the United Kingdom* (TRC Research Paper No. 4) was published in 1966.

#### 2. Cigarette smoking characteristics in the U.K., South Africa, and Australia

Investigations were carried out in the UK, South Africa and Australia on as comparable a basis as possible in order to obtain certain detailed information about cigarettes smoked and the way they were smoked in these three countries. The main facts investigated were the type of cigarettes smoked (i.e. plain or filter-tipped), the lengths and weights of the cigarettes smoked, the types of tobacco used in the cigarettes, the lengths of butts discarded and the lengths of cigarettes consumed, the length of time the cigarettes were alight, the number of puffs per cigarette, and the percentages of cigarette smokers who inhaled moderately or deeply. The results were published in TRC Research Paper No. 5. There was found to be a remarkable similarity of cigarette smoking habits in the countries so far apart.

#### 3. Tobacco consumption in various countries

A report was published (TRC Research Paper No. 6) summarising the figures of cigarette, cigar and other forms of tobacco consumption in 23 countries.

#### 4. Reliability of statements about smoking habits

A report (TRC Research Paper No. 2) by Mr G. F. Todd and Mr J. T. Laws on the reliability of statements about smoking habits had been published in 1958. This report summarised the results of studies of the reliability of statements about current and past smoking habits that had been carried out in 1952 and 1957. A further study was carried out in 1964 in order to obtain information about the errors made in recalling past smoking habits over an average period of 11 years. The results were published in a supplementary report by Mr G. F. Todd (TRC Research Paper No. 2A).

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Section 2. Chemical

Bibliography of tobacco smoke constituents

An annotated bibliography of tobacco smoke constituents (TRC Research Paper No. 3) was published in 1959. The 1st supplement to the bibliography was published in 1960; a second supplement, edited by Mr E. G. N. Berry, was published in 1963; and a third supplement is being prepared.

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# CARCINOGENIC ACTION OF CIGARETTE SMOKE CONDENSATE ON MOUSE SKIN

AN ATTEMPT AT A QUANTITATIVE STUDY

T. D. DAY

*From the Tobacco Research Council Laboratories, Harrington*

Received for publication September 25, 1966

It has been known for some years that when cigarette smoke is condensed and the condensate subsequently dried and stored it forms a "tar"-like substance which when painted on the backs of certain strains of mice gives rise to epithelial tumours (literature reviewed by Wynder and Hoffmann, 1964). Among all the efforts which have been made in recent years to study the carcinogenic properties of cigarette smoke experimentally, this still remains the salient phenomenon: cigarette smoke condensate undoubtedly has the property of a complete carcinogen for epithelial tissue of laboratory animals. The fact that dried cigarette smoke condensate possesses this property has led to the belief that the agents in cigarette smoke condensate principally responsible for its mouse skin carcinogenicity are likely to be stable non-volatile compounds. This belief in turn has formed the basis for the suggestion by some workers (notably Wynder and Hoffmann (1964)) that reduction of total cigarette smoke condensate and reduction of agents in the condensate responsible for mouse skin carcinogenicity might be useful steps to take.

However, up to the end of 1962 when work was started at these laboratories, little account had been taken of the possible contribution to mouse skin tumorigenicity of even semi-volatile components of cigarette smoke condensate. Further, the possibility had not yet been examined that the processes, particularly of drying, involved in preparing smoke condensate for skin application, might have either decreased its tumorigenicity through the destruction of unstable compounds, or alternatively increased this tumorigenicity by the production of carcinogens which were not present in freshly prepared condensate. The effect of storing "tar" for 6 months instead of 1 month had been studied (Wynder, Graham and Croninger 1955), but no work had previously been reported on "tar" less than 1 month old. Neither had an attempt been made by large scale bioassay techniques to identify the important classes of tumorigenic compounds in cigarette smoke. In other words it was not known with reasonable certainty, when the work described in this report was started, whether stable non-volatile constituents of cigarette smoke condensate were important or trivial contributors to its mouse skin tumorigenicity. The general aim of the work to be described was to investigate this question on a scale sufficient to give reliable answers and to investigate further the suggestion (Wynder and Hoffmann, 1959; Day, 1961) that polycyclic aromatic hydrocarbons, or some other neutral constituents, might account for much of the tumorigenic activity of cigarette smoke condensate.

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It is obvious that the mouse skin painting test is unsuitable for detecting the effects of constituents with very low boiling points. These are difficult to isolate and keep in solution in the usual organic solvents at room temperature; it is impossible to treat mice with very cold materials and in any case the natural body heat of the animals would lead to rapid loss of material with a very low boiling point, probably before the skin could absorb it. However, it is possible to compare a condensate containing semi-volatile substances, such as are retained in a low boiling solvent, with one from which these substances have been evaporated. Accordingly it was decided to compare the mouse skin tumorigenicity of acetone solutions of:

- (1) Whole cigarette smoke condensate, applied as quickly as possible after collection while using standard collection traps, hereafter referred to as "24-hour condensate".
  - (2) Whole cigarette smoke condensate which had been evaporated to constant weight and stored for at least one month before application, hereafter referred to as "stored condensate".
  - (3) The residual fraction from whole smoke condensate, after storage for at least one month,
- with
- (4) Unoperated animals and animals treated with acetone only, kept as controls.

Experiments by previous workers in this field had used relatively small numbers of animals, so that statistical analysis of the results had shown significant differences only for large effects. It was further recognised that the yields of tumours from the condensate treatments might be low and that the differences between the treatments might be small. Accordingly, the present experiment was conducted on a scale considerably larger than had previously been attempted, and with a degree of attention to detail not before attained, in order to be able to make the necessary quantitative comparisons acceptably precise.

#### MATERIALS AND METHODS

##### *Experimental design*

A maximum response of about 30% of animals developing tumours was expected at the highest practicable dose. The experiment was therefore planned to enable a difference between 30% tumour rate and 20% tumour rate to be detected at the  $p = 0.02$  significance level in four tests out of five. To give some estimate of the dose-response relationship and to guard against the possibility of choosing the wrong dose, it was planned to divide those animals treated with condensates equally between three dose levels with dose ratios 1 : 2 : 4. A total of about 8000 mice was needed in order to achieve these aims.

Each of the four treatments was divided into three sections. Mice in each of the three sections of the condensate treatments received different doses: mice in one section of the control treatment were treated with acetone alone, mice in the remaining two sections were untreated. The experiment was housed in four animal rooms, using mice supplied at different times: each section was equally represented in each room and in every row of animal boxes in each room. The difference between animal rooms proved to be important, so for most purposes the

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experiment could be regarded as 40 groups of 165 mice each and four groups of 330 mice.

#### Mice

The mice were virgin females of a specific pathogen-free, albino strain supplied by Dr. W. G. Davey of the Pharmaceuticals Division, Imperial Chemical Industries Ltd. This strain, as the present paper shows, combined resistance to nicotine with sensitivity to skin carcinogens. Male mice were not used, being more pugnacious and so liable to skin damage. The mice, 4-6 weeks old on receipt, were transferred to sterile boxes and allowed to acclimatise for one month before skin applications were made. Four groups of 2100 animals, received at intervals of three months, were used. Of each group, a total of 1980 were used for treatment, 100 for calibration tests with standard carcinogens as described below, and the remainder for replacement of any animals found to be unsuitable; as for instance being unduly small or having been injured in transit. Mice were not replaced after skin treatment had commenced, and treatment was continued until the animals died or had to be removed due to illness.

Each group of 1980 mice was housed in a separate air-conditioned room, kept at 20-21°C, the room being to separate the experiment into four equal units in order to limit the spread of any adventitious infective disease. In the event, no epidemic disease occurred and the death rates of the colonies in each of the four rooms although not identical were similar.

Mice were identified by ear punching and kept on sterile sawdust, three in a box in sterilised galvanised iron boxes, being transferred to clean boxes twice a week. Drinking water in sterile bottles and heat sterilised (30 min. at 63°C) Oxoid Breeding Diet pellets were provided *ad libitum*.

A skin area 10 cm wide from nape of neck to base of tail of each mouse was kept clipped. Sesame oil (generally regarded as non-carcinogenic and non-promoting) was used as clipper lubricant.

#### Dosing procedure

A preliminary toxicity test of nicotine-containing condensate was undertaken in order to ascertain the highest practicable dose-level for long-term application. Dosing by the standard procedure described below was continued for six weeks; acute toxicity was estimated by observing whether the animals were ill immediately after dosing, and chronic toxicity by observing whether the animals gained weight. The results indicated that a top level equivalent to 100 mg. non-volatile whole smoke condensate (as defined below) per application was suitable, after habituation, for long-term treatment with acceptably low mortality. Acetone solutions of test materials, prepared as described later, were therefore applied at dose levels equivalent to 100, 50 and 25 mg. of non-volatile whole smoke condensate per application, the highest dose level being commenced only after habituation for one month at 75 mg. dose per treatment.

Skin applications were made three times a week, with a foot-controlled automatic pipette, delivering for all treatments a standard volume of 0.3 ml. through silicone rubber tubing; pipettes were checked and re-calibrated if necessary daily. The standard volume of fluid was applied evenly on the clipped area.

The reaction to skin carcinogenesis of a sample from every batch of mice

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received at three-monthly intervals was checked by treatment with acetone solutions (0.1% w/v) of 1,2,5,6-dibenzanthracene and 3,4-benz(a)pyrene, 0.019 ml. of solution being applied three times per week. Applications to one-half of each sample of mice was stopped after 10 weeks, treatment of the remainder continuing for 25 weeks. All these animals developed tumours in a few months, no great differences being observed between the batches.

#### *Pathology at death and diagnosis of tumours*

A full post mortem examination was performed on each mouse, whether bearing skin tumours or not, and a suitable sized piece of tissue was taken from any organ showing abnormality. Slides and post mortem descriptions were interpreted by the same pathologist throughout. All tumours were examined histologically and their nature recorded, a clear distinction being made between tumours occurring in the treated area of the skin and elsewhere. A total of 45 animals were recorded as dying from nicotine poisoning; their records were eliminated from all results. A summary of the pathology recorded at death for the remaining mice is given in Table I.

TABLE I.—*Pathology Recorded at Death*

All 784 mice in the experiment (4 × 1980 less 45 deaths due to poisoning)

Predominant pathology at time of death	Per cent of all mice with this pathology
Hepato-nephritis	23.0
Malignant lymphoma	22.6
Inflammation of uterus and other miscellaneous inflammation	12.2
Dermatitis and skin sepsis	8.2
Malignant tumours (not on painted area and including sarcoma)	6.7
All haemorrhages	6.7
All benign tumours (haemangiomas, papilloma of painted area and adenomas other than of lung)	5.0
Due to experiment (cause not determined, etc. but not nicotine poisoning)	4.4
9. Adenoma of lung	3.6
10. Miscellaneous (general)	3.2
11. Malignant tumours on painted area	3.0
12. Staphylococcal osteomyelitis	0.9

Apart from skin sepsis, which was more common in the high dose groups, the pathology was not particularly related to treatment and the incidence of tumours, other than those of the painted area, did not vary significantly with dose or treatment. Any animal considered at any time by the animal superintendent to be irrecoverably ill was killed, as were mice which escaped from their boxes.

For cancers of the skin in the treated area, particular attention was paid to making the diagnosis of malignancy independent of subjective factors. The strict criterion of malignancy adopted was penetration by the epidermal tumour between the muscle fibres of the panniculus carnosus ("muscle-infiltrating carcinoma") as shown in Fig. 1. Tumours which had the subjective appearance of

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infiltrating cancer but which did not fulfil this strict criterion of muscle penetration were separately classified ("carcinoma not infiltrating muscle"). The rare occurrence of a subcutaneous sarcoma or haemangi endothelioma in the treated area was also noted.

The date of first appearance of a papilloma was noted on a record card kept for each animal throughout the experiment. Tumour-bearing animals were normally killed when the tumour appeared to have become malignant, as judged by the pathologist pinching up the skin and assessing the degree of infiltration on its under surface. The date of death was then recorded as the date of appearance of a carcinoma, if one was found by histology.

#### *Cigarettes*

Plain cigarettes (length 70 mm., circumference 25.3 mm., average weight 1.125 g.) were specially manufactured from a composite blend of flue-cured tobacco representing the major plain cigarette brands smoked in the United Kingdom, packed in batches of 50 in vacuum-sealed tins and stored at 4 °C. before use.

#### *Smoking procedure*

The automatic smoking machine used operates by connecting each of 24 cigarettes, secured in holders situated round a revolving disc, in turn to a source of vacuum, the unlighted end of each cigarette being open to atmosphere between puffs (Fig. 2). Cigarettes were lighted by an electrically heated coil. When individual cigarettes had reached an estimated butt length of 20 mm., the butts were removed and replaced with fresh cigarettes.

Automatic smoking constants were chosen to simulate the manner used by the average cigarette smoker in the United Kingdom (Bentley and Burgan, 1961):

Puff volume, 25 ml.; Puff duration, 2 seconds;

Puff interval, 1 minute; Butt length, 20 mm.

#### *Whole smoke condensate (WSC)*

Cigarette smoke was collected in four glass traps connected in series (Fig. 3), cooled in acetone/crushed solid carbon dioxide. Traps 3 and 4 each contained glass helices (100 mm. diam. single turn). On completion of smoking, traps were allowed to attain room temperature, condensed smoke was washed from the traps and connecting tubes with acetone (about 900 ml.), the washings filtered through glass wool and an aliquot of the combined filtrate taken to check non-volatile whole smoke yield by determination of nicotine. The average yield of nicotine was 1.61 mg./cigarette, range 1.30–1.91 mg./cigarette.

#### EXPLANATION OF PLATES

FIG. 1a.—Malignant infiltration of the subcutaneous tissue by epidermal cancer cells, but these have not yet penetrated the muscle. H. and E.  $\times 80$ .

FIG. 1b.—Epidermal cancer cells which have infiltrated between the fibres of the panniculus carnosus muscle. This is the usual criterion for the diagnosis of malignancy. H. and E.  $\times 190$ .

FIG. 2.—Smoking machine.

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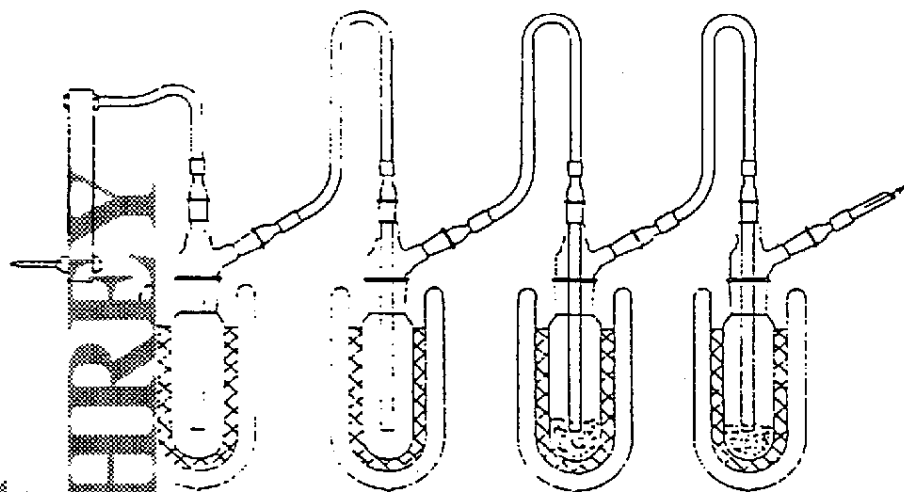


Fig. 3. Smoke condensation system. The arrow on the right shows the exit to the smoking machine via a cotton wool filter. The two right hand traps contain Fenske helices (single turn) and all four Dewar flasks are filled with solid carbon dioxide and acetone.

#### *Non-volatile residue smoke condensate (NVWSC)*

Solvent was removed from the acetone solution of WSC in a weighed flask, using a rotary evaporator on a boiling water bath with a water suction pump operating at a vacuum of 10 inches of mercury; evaporation was continued until the non-volatile residue attained constant weight. The average yield was 21.5 mg. cigarette range of 17.7-24.8 mg./cigarette. All doses of all materials applied to animals were expressed in terms of the weight of NVWSC determined in this way, each individual dose, irrespective of weight, being delivered in the standard volume (0.3 ml.) of solution. The average yield of NVWSC over a four-week period was used to compute the number of cigarettes to be smoked for both 24-hour condensate solutions and for the stored condensate and neutral fraction required during the subsequent four-week period.

#### *Stored condensate*

NVWSC collected over four weeks was combined, stored at  $-29^{\circ}\text{C}$ . for a further four weeks, dissolved with constant stirring in acetone/water (9 : 1 v/v) and the solution diluted to the appropriate volume with the same solvent.

#### *24-hour condensate*

The acetone solution of WSC from the calculated number of cigarettes was sampled for nicotine content to check the actual WSC yield and concentrated in a rotary evaporator (water bath temperature  $40^{\circ}\text{C}$ . and the full vacuum of a water suction pump), until it reached the concentration required for application at the highest dose rate. Aliquots of the concentrate were then taken for dilution with acetone/water (9 : 1 v/v) to provide solutions for the two lower dose levels.

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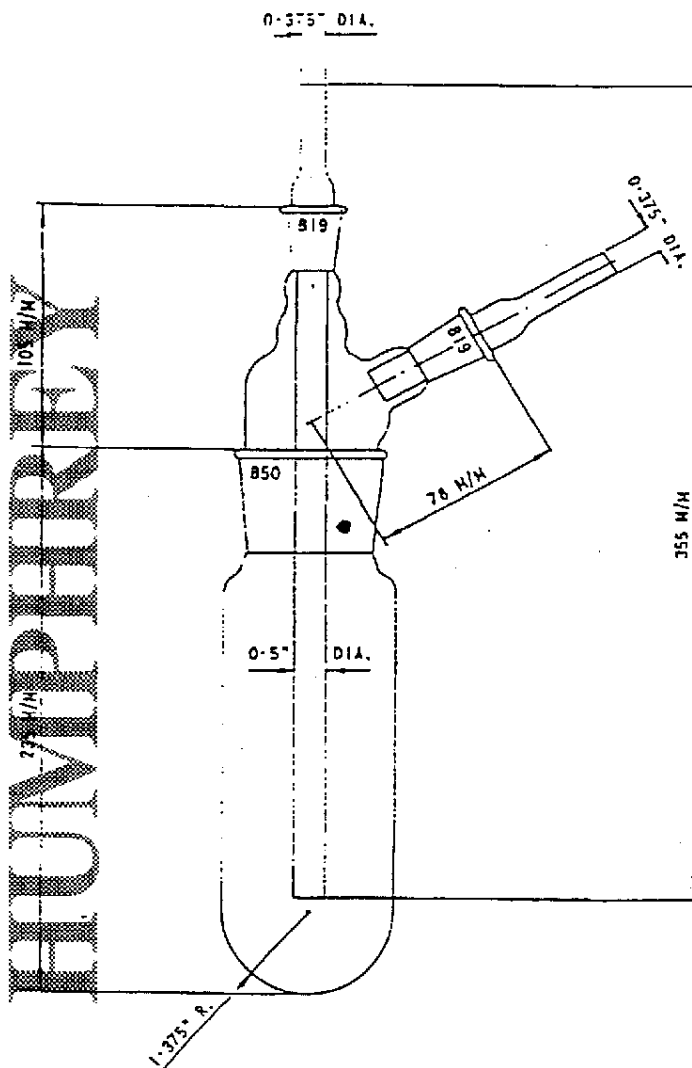


FIG. 3b.—Details of Trap.

#### Neutral fraction

Redistilled peroxide-free Reagent Grade diethyl ether was treated with sodium to remove fluorescent material. The smoke condensate from a known number of cigarettes (about 2000) was washed from the traps into a separating funnel with ether (1 l.) and hydrochloric acid ("Analar" 2 N, 900 ml.). the mixture was shaken, allowed to separate and the aqueous layer drawn off into a second separating funnel. After further extractions with hydrochloric acid (2 × 200 ml.,

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5 : 100 ml.), the combined acid extracts were washed with ether (2 : 100 ml.), and the ether washings returned to the ether solution left in the first separating funnel. The combined ether solution was then vigorously shaken with potassium hydroxide solution (3% w/v, 200 ml.), allowed to stand for 1½ hours, the aqueous layer drawn off and further extractions carried out with potassium hydroxide solution (1 : 200 ml., 5 : 100 ml.); little emulsification occurred after the first extraction and the two layers separated without difficulty. The final ether solution was dried (anhydrous magnesium sulphate), filtered and the solvent removed in a rotary evaporator (water bath at 40° C. and a water suction pump).

The average yield of neutral fraction was 6.68 mg./cigarette, range of 5.00–7.88 mg./cigarette; the condensate yield of each batch was checked by estimating the nicotine content of a suitable aliquot of the combined acid extracts. Batches of neutral fraction produced in a four-week period were combined and the corresponding NWSC equivalent calculated from the total number of cigarettes smoked and the average yield of NWSC obtained for stored smoke condensate over that period of four weeks. For dosing, the combined neutral fraction was dissolved in nicotine water (9 : 1 v/v).

#### Nicotine assay

Nicotine content was measured on aliquots of the condensate obtained from every individual smoking, to check on smoking performance and extraction efficiency, by the method of Willits, Swain and Connelly (1950), as modified by Laurence and Hargill (1958).

#### DISCUSSION OF RESULTS

Table II shows for all animals the percentage rates (100 × No. affected/Initial No.) for death and incidence of three classes of tumours at four times during the experiment. No allowance has been made in these results for differences in mortality between treatments, and it can be seen that there are quite large variations in mortality from one treatment to another. To give a fair comparison of the treatments, independent of their toxicity, some form of age standardisation must be done on the assumption (which seems unavoidable in experiments of this type) that animals which develop tumours would not, in the absence of treatment, have lived longer or died sooner than the others. The age standardised results, calculated in the way described in the Appendix are shown in Table III.

In order to attain the objectives outlined earlier, it was necessary to discover if the data would permit a valid comparison to be made of the tumorigenicities of the three materials under test. It was not the intention in planning the experiment to investigate the mechanisms of carcinogenesis, for example the relative contributions in each material from tumour initiators and tumour promoters, but unless the three materials act in broadly similar ways there can be no valid basis for comparing their relative tumorigenicities. Graphical representation of the data, exemplified by Fig. 4, 5 and 6, in which no account is taken of the statistical confidence limits of the points, suggested an anomaly in that at the low and medium dose levels stored condensate and neutral fraction appeared to have similar activities, each different from 24-hour condensate, whereas at the high dose level stored condensate and 24-hour condensate appeared to have similar activities, each different from neutral fraction. The statistical analysis, which is

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TABLE II—Unstandardised Percentage Response

Treatment and Dose level	Age	Deaths Weeks				Tumour-bearing animals Weeks				Carcinoma-bearing animals Weeks				Animals bearing infiltrating carcinomas Weeks			
		56	80	104	128	56	80	104	128	56	80	104	128	56	80	104	128
Controls, Untreated	25-70	61-03	92-73	100-55	0-00	0-00	4-29	7-73	0-00	0-00	0-00	0-00	0-00	0-00	0-00	0-00	0-00
Controls, Acetone	25-04	61-70	92-43	99-39	0-00	0-00	0-76	0-00	0-00	0-00	0-00	0-00	0-00	0-00	0-00	0-00	0-00
Neutral fraction, low	28-07	61-34	92-87	99-70	1-37	3-31	5-92	6-98	0-00	0-00	1-06	1-21	0-00	0-30	0-40	0-46	0-46
Neutral fraction, med.	24-39	61-06	92-88	99-70	1-06	7-58	13-61	16-06	0-30	1-06	3-70	4-09	0-15	0-35	1-82	1-82	1-82
Neutral fraction, high	28-64	68-04	95-45	100-00	5-20	11-85	21-30	24-07	0-45	3-70	7-12	7-73	0-30	2-73	4-79	5-15	5-15
Stored condensate, low	24-89	58-73	92-41	99-85	0-01	3-19	5-31	5-77	0-00	0-30	0-76	1-06	0-00	0-15	0-46	0-61	0-61
Stored condensate, med.	29-36	63-64	92-12	100-00	2-42	6-82	12-27	13-33	0-00	1-67	3-33	4-39	0-00	0-76	1-52	1-82	1-82
Stored condensate, high	30-20	73-04	97-58	100-00	14-55	27-12	32-27	32-58	1-52	8-48	13-94	14-85	1-06	5-30	9-24	9-70	9-70
24 hr. condensate, low	23-33	57-73	91-07	99-70	2-27	6-39	12-58	13-33	0-30	1-67	3-94	4-85	0-30	1-06	2-73	3-33	3-33
24 hr. condensate, med.	30-30	70-15	98-07	100-00	8-03	19-09	25-00	25-91	0-61	4-39	9-39	10-30	0-45	2-88	4-85	5-30	5-30
24 hr. condensate, high	32-36	71-20	99-19	100-00	13-75	26-51	31-88	31-88	0-81	4-53	10-26	11-17	0-32	2-91	5-18	5-55	5-55

TABLE III. *Standardised Percentage Response*

Treatment and Dose level	Deaths Weeks				Tumour-bearing animals Weeks				Carcinoma-bearing animals Weeks				Animals bearing infiltrating carcinomas Weeks			
	56	80	104	128	56	80	104	128	56	80	104	128	56	80	104	128
Controls, untreated	25.17	58.00	86.05	90.37	0.23	0.01	1.44	1.67	0.00	0.00	0.08	0.08	0.00	0.00	0.00	0.00
Controls, Aroclor	21.73	59.61	87.10	90.11	0.00	0.30	0.76	0.76	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Neutral fraction, low	28.68	63.58	90.90	94.54	1.37	3.31	6.22	6.68	0.00	0.70	1.06	1.06	0.00	0.70	0.40	0.40
Neutral fraction, med.	23.79	57.73	80.82	92.42	1.06	7.58	15.15	15.76	0.30	1.06	3.04	3.79	0.00	0.45	1.67	1.67
Neutral fraction, high	28.33	70.61	102.88	108.64	5.45	17.12	27.27	27.73	0.45	3.04	8.48	8.64	0.00	2.73	5.45	5.76
Stored condensate, low	21.28	55.51	84.97	88.92	0.61	2.88	5.31	5.61	0.00	0.30	0.76	0.76	0.00	0.45	0.40	0.40
Stored condensate, med.	25.91	61.85	92.42	98.91	2.12	6.07	11.97	13.03	0.00	1.67	3.48	3.79	0.00	0.76	1.67	1.82
Stored condensate, high	30.45	79.24	121.97	128.48	15.91	33.79	47.12	48.61	1.52	9.55	20.30	21.97	0.00	5.91	14.09	14.85
24 hr. condensate, low	22.58	52.88	82.58	86.97	2.27	8.91	11.97	12.58	0.30	1.30	3.48	3.79	0.00	1.06	2.42	2.73
24 hr. condensate, med.	30.76	73.04	109.70	116.52	8.48	22.88	33.79	35.76	0.61	5.00	11.67	13.61	0.00	3.48	5.45	6.36
24 hr. condensate, high	33.66	77.18	128.04	132.69	15.37	34.63	50.49	50.49	0.81	5.02	16.99	20.71	0.00	3.40	7.61	9.55

All treatments applied three times a week until death.

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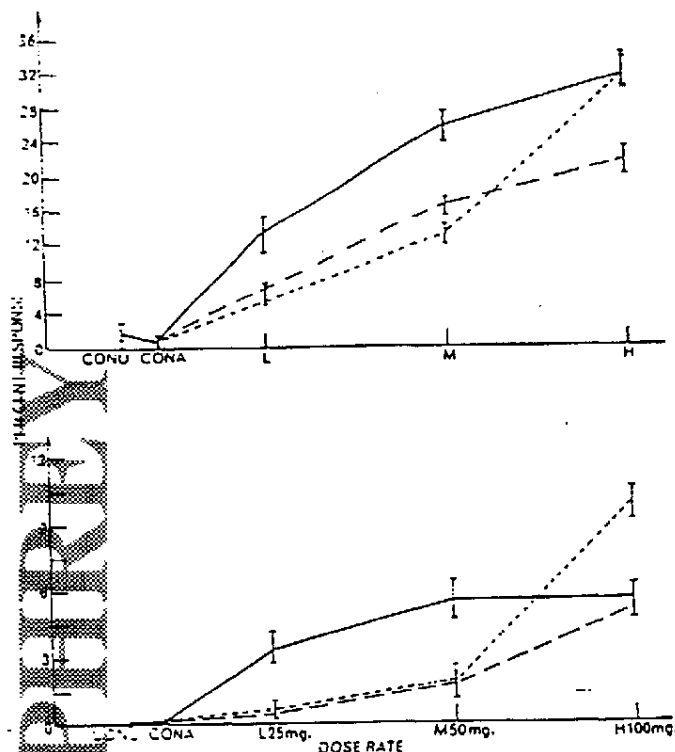


FIG. 4. Dose-response Graphs. Unstandardized. 128 Weeks. Upper: All Tumours.

Lower: Infiltrating Carcinomas.  
 Neutral Fraction  
 Stored Condensate  
 24 Hour Condensate  
 Doses measured in mg. F.A.S. per application

described in the Appendix, showed that the response curves for every group of animals could be fitted by a lognormal distribution over time of the tumorigenic force, which is defined as the number of new tumour-bearing animals found in a short period divided by the number of tumourless animals alive in the group at the start of the period. This distribution has three parameters, its standard deviation, which was found to be unaffected by treatment or dose, the proportion of animals which would never develop tumours however long they lived, and the mean of the distribution, which measures the average time from the start of treatment to the appearance of a tumour (the mean tumour-induction time). These last two parameters were found to be highly correlated, long tumour-induction times appearing with large proportion never developing tumours, so that either can be used as a measure of response to the treatment. With the three condensates used in this experiment the mean tumour-induction time falls by about 25% when the dose is doubled, over the range of doses used, and this relationship can be used

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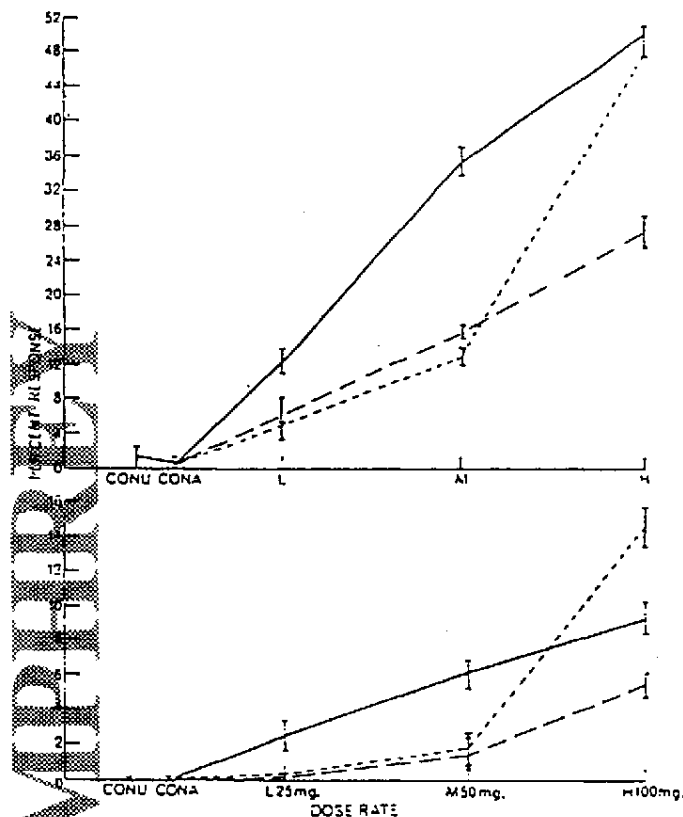


FIG. 3.—DOSE-Response Graphs. Age Standardised. 125 Weeks. Upper: All Tumours. Lower: Infiltrating Carcinomas

Neutral Fraction ———  
 Stored Condensate - - - - -  
 24 Hour Condensate ·····  
 Doses measured in mg. F.A.S. per application

to relate the differences between condensates to the change in dose which would be needed to give equal responses, in the same way as in a toxicity bioassay.

Table IV shows the relative tumorigenicities calculated from the mean tumour-induction times and the proportion never developing tumours, and also the values calculated from the standardised rates. These latter values are more complicated because they contain the statistically significant anomaly in the stored condensate results (described fully in the Appendix) which was not apparent in the mean tumour-induction times.

The first important feature of this table is the uncertainty in the relative tumorigenicity even working with a total of 8000 mice. For example, the ratio for 24-hour condensate and neutral fraction at the 95% confidence limit, lies somewhere between 1.5 and 2.1. This illustrates strikingly how difficult quanti-

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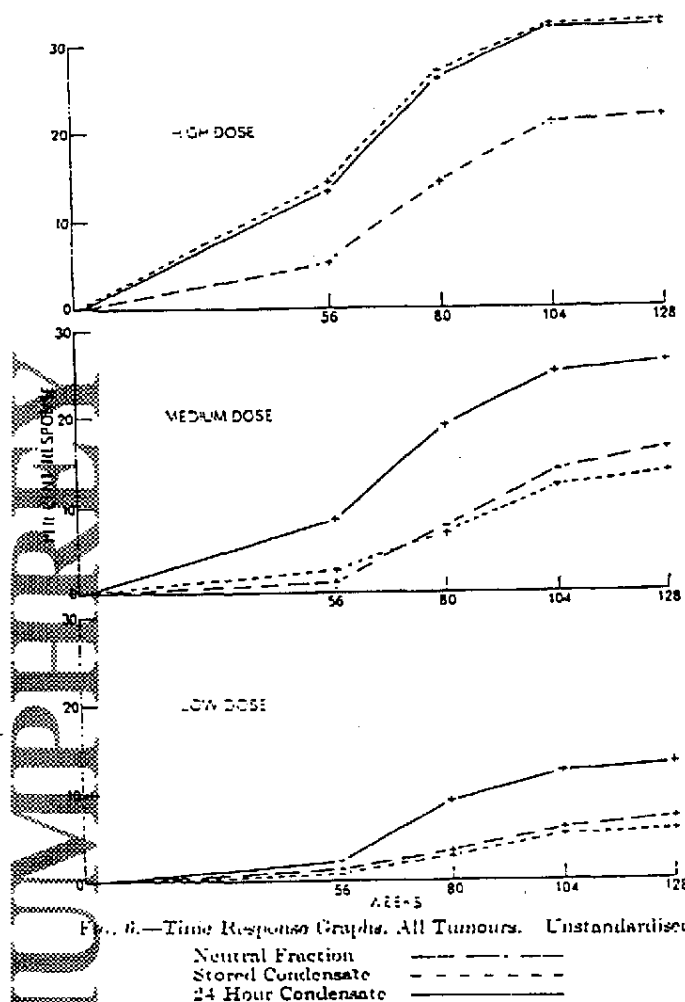


Fig. 6.—Time Response Graphs. All Tumours. Unstandardised.

Neutral Fraction ———  
 Stored Condensate - - -  
 24 Hour Condensate . . .

tative work in this field and it may be noted that had the experiment been carried out on only about 1/12 of this scale with 50 animals on each dose level, or 600 in the whole experiment, then the rates of tumorigenicity for 24-hour condensate and neutral fraction would have had confidence limits of at least 1.0 to 3.0 at the 95% level.

Within the limits used the tumorigenicity of each material increased linearly with the log dose so that even at the highest dose used there was no evidence that the point of saturation of the tumorigenic action was near. Not unexpectedly, the mean tumour-induction times for carcinomas and non-infiltrating carcinomas were longer than those for all tumours but it is of considerable interest that the relative tumorigenicities of 24-hour condensate, stored condensate and neutral

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TABLE IV.—*Relative Tumorigenicities*

From all tumours, all carcinomas and infiltrating carcinomas combined.

Dose levels	Denom.	From mean tumour-induction times and proportions of animals never with tumours			From standardised tumour rates at 125 weeks		
		Neutral fract.	Stored cond.	24-hour cond.	Neutral fract.	Stored cond.	24-hour cond.
25, 50	Neutral fract.	1	1.10	1.77	1	0.89	1.55
100						20%—16% 2.06—2.22	16%—13% 1.94—1.66
25, 50	Stored cond.	..	1	1.59	..	1	1.94
100						1	20%—16% 1.94—1.66

Symmetric conf. limits  $p = 0.95$  are  $-75\%$ — $+11\%$  of the ratioThe "% figures are symmetric conf. limits  $p = 0.95$ 

fraction were found to be in the same ratio using in turn each of these three tumour types, as can be seen from Table V. This suggests that the three condensates act in broadly the same way on the test animal and that the relative tumorigenicities which we have chosen to refer to intrinsic properties of these condensates.

TABLE V.—*Relative Tumorigenicity*

From mean tumour-induction times

	All tumours	Carcinomas	Infiltrating carcinomas
Stored Neutral	1.07	1.08	1.15
24-hour Neutral	1.07	1.03	1.09
24-hour Stored	1.58	1.50	1.47
95% Conf. limits (equal tails)	-29%—+21%	-67%—+30%	-75%—+37%

As stated earlier, it had often been surmised that non-volatile neutral components of cigarette smoke are responsible for the tumorigenicity of the smoke condensate, but in the present experiments it could only be considered as an inspired guess. Our results, using 95% one tail confidence limits, suggest that non-volatile neutral components account for something more than 50% of the tumorigenicity of 24-hour cigarette smoke condensate, and 80% of that of stored cigarette smoke condensate as defined in this report. It then follows that the compounds responsible for this effect are stable after collection for several weeks and are not affected by moderate heat treatment and chemical manipulation. It is unlikely that they are produced as artifacts in the process of making stored condensate.

The results certainly suggest that 24-hour condensate is more tumorigenic than stored condensate. While neither material contains what is usually termed the volatile fraction, there must be substances in 24-hour condensate which are lost or modified when evaporation is carried out to dryness: chemical analyses of these condensates are however not informative because at this stage we do not know to which of the large number of constituents attention should be directed. The

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results provide no evidence to decide whether any part of the activity of 24-hour condensate is due to its processing or storage.

In practical terms, an important feature of these results seems to be that in relation to mouse skin they show that there are stable non-volatile neutral carcinogens in cigarette smoke condensate which are worth serious attention and which in particular merit investigation by detailed fractionation.

## SUMMARY

The mouse-skin carcinogenicity of freshly prepared cigarette smoke condensate was compared at equivalent dose rates with that of a condensate which had been evaporated to constant weight on a boiling water bath and subsequently kept for several weeks, and with that of a neutral fraction of the condensate. The carcinogenicity in terms of several different response measures was linearly related to the logarithm of the dose, and the response to the three different materials was broadly similar, the freshly prepared condensate being more active than the other two materials used.

The results provide evidence that non-volatile neutral components of cigarette smoke contribute substantially to the mouse skin carcinogenicity of whole 24-hour old cigarette smoke condensate, as defined in this report and, for the first time, that the compounds responsible for this effect are stable from 24 hours after collection for several weeks, and that these compounds were not produced as artifacts in the processing leading to stored condensate. The substances responsible for the additional carcinogenicity of 24-hour old condensate have not been identified.

I should like to thank the scientists of member companies of the Tobacco Research Council for their advice and help in this experiment, and particularly Mr. S. P. ... suggesting and carrying out the statistical analyses in this report and for preparing the statistical Appendix. The experiment also owes much to Mr. ... who was responsible for the preparation of the condensates, to Mr. D. V. D. Thoroughgood who was responsible for the animal husbandry, and to those many other technicians whose efforts have made this work possible.

## APPENDIX

## STATISTICAL CONSIDERATIONS

W. S. PAIGE

Several techniques are described in the literature for the analysis of mouse tumour experiments. The earliest is due to Twort and Twort (1933) who used three methods. The first two depend upon comparisons of cumulative percentages of tumours with a standard curve, and the third estimates the time at which 25% of the animals would have developed tumours, in the absence of mortality, using a crude approximation to the tumour expectation of animals which died during the experiment. Irwin and Goodman (1946) compared these measures with the standard actuarial calculations, which were also used by Palmes, Orris and Nelson (1962) and Blanding *et al.* (1951) and recommended the use of expectation of tumourless life, when there is no mortality, as the most clearly interpretable

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measure. In our terms this is the mean tumour induction time. They considered that the date at which a particular tumour rate was reached was not in general a useful measure of response.

Bryan and Shimkin (1941) described the application of standard bioassay techniques to this problem, but they were concerned mainly with potent carcinogens and did not use efficient techniques for comparison in the presence of natural mortality. They also gave analyses of tumour-induction times (under the name "latent period") showing that in some cases they were independent of dose. They advocated the use of the ED50, the dose which produced 50% response, for the comparison of different treatments; and suggested that the mean tumour induction time for this dose was a useful parameter. Their formula for the time at which an experiment could be safely terminated shows the necessity of continuing experiments with weak carcinogens until all the animals are dead. Lea (1945) criticised this paper by Bryan and Shimkin for its lack of emphasis on techniques for eliminating differences in mortality. He suggested, for experiments with continued or persistent treatments, a maximum likelihood procedure for estimating a lognormal distribution over time of the tumorigenic force, which made full allowance for mortality. This method, however, suffered from the disadvantage that it assumed that all animals would develop tumours if they lived indefinitely, which does not seem to be justified with control groups or weak carcinogens.

Blanding *et al.* (1951) described a technique in which an effective tumour rate is plotted on log probability paper to estimate the time at which the rate will be 50%. This effective rate is the number of animals which have borne tumours divided by the original number less deaths without tumours. This rate is neither an uncorrected rate nor a standardised rate, and it is difficult to use in any theoretical model of tumour incidence. They also recommend the use of a tumour potency the reciprocal of the time-to-50%-rate, but this does not seem nearly as suitable a measure as the equivalent dose considered below for use with large experiments on weak carcinogens, since it depends on the stability of the animal population used, and its variance depends upon the mean.

Wynder (for example, Wynder and Hoffmann, 1959) and other workers have used the number of tumours or the number of tumour-bearing animals at a particular date to compare treatments; but this method is open to objections, as described below, if the different treatments have different mortality rates. However, this approach was used as the starting point in an attempt to develop an improved analysis.

#### ANALYSIS OF RATES

A number of different analyses have been done on our data, but they fall into two main types: the first deals with "tumour rates" defined as the number of tumour-bearing animals observed to date in a group divided by the number of animals in the group at the start of treatment. This is a cumulative measure which smooths out statistical fluctuations, but causes difficulties when independent statistical tests are needed for results at different stages of the experiment. The second group of analyses deals with the "tumorigenic force" defined as the number of new tumour-bearing animals observed in a group during a short time interval divided by the number of tumourless animals alive in the group at the start of that

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interval. The observations for different intervals are here uncorrelated, but suffer from large statistical fluctuations.

The simplest analysis which can be done is a comparison of the tumour rates at a given time for groups of animals under different treatments. In the case of toxic treatments (and 24-hour smoke condensate is somewhat toxic in the doses used in this experiment, since the mortality from all causes is higher under this treatment) there are two courses open. Firstly, the number of tumours which was actually observed can be used as it stands. If this is done a treatment which is toxic but has the same capacity for producing tumours as a non-toxic treatment will show a smaller number of tumours than the non-toxic treatment because the number of animals at risk at any time will be smaller if some have been poisoned. Alternatively, it can be assumed that the poisoned animals were a random sample of the whole. Then some form of correction can be applied for the varying mortalities to give standardised numbers of tumours and an analysis can be done of the response excluding the toxic effect. In order to do age standardisation by any technique it is necessary to assume that the expectation of life for those animals which develop tumours would, in the absence of treatment, be the same as that of the remainder of the group. This assumption is basic also in the analysis of tumorigenic data, since the denominators in all the fractions, for different treatments and for different times, must be comparable.

Detailed analyses of tumour rates and death rates were carried out at 24-week intervals from 24 weeks to 128 weeks from the start of treatment. Table II above shows, for all four rooms taken together, the average un-standardised rates at the four selected times, and Fig. 4 shows the results for 128 weeks graphically. Table III and Fig. 5 show corresponding age standardised rates. The age standardisation used the direct method (Yule, 1934), taking as its standard population the mean mortality rate for all animals in the experiment, which reduces the corrections needed almost to the minimum possible. For each week in turn the age-specific rate for a group of animals was applied to the proportion surviving of the standard population and these results were accumulated to give a standardised rate up to a given age for that group. To show the magnitude of these corrections the standardised death rates are also shown. In a group with greater than average mortality the standardised mortality will of course exceed 1 towards the end of the experiment. It was hoped originally to calculate mean tumour-induction period by the actuarial method (Irwin and Goodman, 1946; Blanding *et al.*, 1951) but the observed rates were all well below 50% so the extrapolation involved made the results unreliable.

A preliminary analysis of deaths showed that as well as differences between the treatments there were several anomalies in the mortality curves for different animal rooms. This is shown in Table VI, which records the cumulative numbers of deaths. The most significant feature is the excess of deaths in Room 2 about week 70. This was due to animals being killed in Room 2 at that time on account of a number of different types of pathology, following a change in the standard of illness applied as a criterion for removal of animals. Similar but much smaller anomalies were found in other rooms at other stages in the experiment but no significant anomalies were found between groups of mice in a room. It was therefore necessary to keep the four animal rooms separate throughout the analysis (though the tables presented here give results for all four rooms together for brevity).

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TABLE VI.—Cumulative Number of Deaths (All Treatments)

Treatment Week	Room			
	1	2	3	4
56	512	537	531	551
72	944	1134	973	1000
88	1464	1589	1522	1501
104	1833	1890	1851	1851

Since there are anomalies in rates between the animal rooms, and because the corrections involved in standardising were quite large (as inspection of Table III shows) significance tests between treatments based upon binomial distribution theory, including the  $\chi^2$  test, are invalid. Analysis of variance, however, does not depend upon the exact fit of the data to particular distributions, and is robust under deviation from distribution assumptions, so it can validly be applied to either the standardised or the unstandardised data.

## ANALYSIS OF VARIANCE

A preliminary inspection showed that the control groups, having few or no tumours, gave different variations between rooms from the treated groups. The analysis of variance was therefore split into two parts:

*A Controls* Two types of control (untreated and acetone treated) - 4 rooms.

*B Treated* 9 treatments - 4 rooms. The 9 treatments were further divided into 3 types of condensate - 3 doses, the dose relationship being expressed as linear and quadratic terms. The between-rooms term, with 3 degrees of freedom, was subtracted from the residual as a "block effect".

The complete analysis of variance is shown in Table VII.

TABLE VII.—Analysis of Variance  $\text{Sin}^{-1}(\text{SQRT}(R))$ 

$R$  = standardised rate, all tumours 104 weeks.

Source	df	SS $\times 10^4$	Mean square $\times 10^4$	F
Total	43	250.55	5.83	—
Between controls	1	39.3	39.3	2.53
Within controls	6	93.1	15.5	1
Control treated	1	9359.2	9359.2	—
Dose linear	1	10712	10712	436
Dose quadratic	1	87.3	87.3	3.55
Condensate	2	2312	1156	47.0
Dose $\times$ cond.	4	1064	266	10.5
Within treat.	24	591	24.6	1.00
Rooms	3	767	255.6	10.4

Analysis of variance cannot be done directly on the rates, since their variance depends upon the mean; the results must be transformed. The usual transformation for rates (Bartlett, 1947) is the inverse sine of the square root of the rate; but since ratios were required an analysis of the log rates was also done. The square root transformation appropriate for Poisson data was tried on some sets of results

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with no rates above 10%. These analyses showed little difference from the inverse sine results and were used only to confirm the findings.

The most striking feature of Table VII is the extreme linearity of the dose effect. In 24 analyses (4 times  $\times$  3 tumours  $\times$  standardised or not) only four quadratic terms (for all tumours, 56 and 104 weeks) were significant at the  $p = 0.05$  level.

The other features of the analysis are shown best by the table of means, and that corresponding to Table VII is shown in Table VIII. As would be expected from Table VI, the difference between rooms is attributable to Room 2.

TABLE VIII.—Means from Analysis of Variance  $\text{Sin}^{-1}(\text{SQRT}(R))$

<i>R</i> = standardised rate, all tumours 104 weeks					
Controls:	Untreated	Acetone			
	0.119	0.075	C.L.	0.049	
Treatments:					
Dose:	Low	Med.	High	Mean	
Neutral fraction	0.248	0.398	0.548	0.397	
Stored condensate	0.228	0.347	0.756	0.444	
24-hr. condensate	0.350	0.618	0.789	0.586	
Mean	0.275	0.454	0.695	0.476	
C.L. individuals	0.049		row col. means	0.028	
C.L. $p = 0.05$					
Room	1	2	3	4	
	0.442	0.555	0.461	0.445	C.L. 0.032
C.L. $p = 0.05$					denotes confidence limits at 95% level.

The interaction of Dose and Condensate is seen to be mainly due to the high dose treatment of stored condensate. The two lower doses show responses very similar to neutral fraction, while the high dose response is considerably higher than that for neutral fraction. This pattern is visible in all the analyses. It has been called the stored condensate anomaly.

There is a corresponding effect, in the opposite sense, in 24-hour condensate, where the top dose response is markedly low. This is not significant in many of the standardised analyses, but it is very significant in the unstandardised tables. It is thought that this is due to the toxic effect of large doses of 24-hour condensate.

Table IX summarises these effects in a number of analyses of variance in columns giving the dose effect, expressed as the increase in response for doubled dose, the differences between condensates, the difference between treated and control and the stored condensate anomaly expressed as difference observed—expected on linear increase. A few values are given for the log analysis, but this was little use for the carcinoma results since it is unsatisfactory with counts below about 5 individuals.

The great difficulty with the results in Table IX is to find some way of combining data for different times. It was clear from these results, however, that the response differences, on all the scales tried, varied with time, and it would be necessary to use the dose-response relationship to convert differences back into equivalent dose ratios.

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TABLE IX. Analysis of Variance Estimates of Effects

Ratio for	To week	Doubled Dose eff.		Difference 24 hr. post.		Difference Stored neut.		Difference treat-control		Stored anomaly (see p. 73)		F Ratio for rooms (24, 30)
		Mean	C.L.	Mean	C.L.	Mean	C.L.	Mean	C.L.	Mean	C.L.	
Unstandardised												
All tumours	50	11.4	1.4	11.4	2.4	11.4	2.4	11.4	2.4	7.9	5.4	0.02
	80	11.0	1.4	11.4	2.7	11.4	2.7	11.4	2.7	8.2	4.0	0.30
	104	11.2	1.4	11.4	2.7	11.4	2.7	11.4	2.7	6.6	4.6	3.64
	128	13.6	1.8	11.4	3.0	11.4	3.0	11.4	3.0	6.6	5.4	1.53
All carcinomas	50	3.5	1.5	3.2	3.0	1.2	3.0	4.3	2.4	4.7	4.6	0.52
	80	8.3	1.0	7.4	3.2	4.8	3.2	14.8	2.4	5.6	4.6	0.26
	104	10.1	1.3	8.0	2.7	2.4	2.7	21.0	2.7	6.9	4.0	1.96
	128	9.7	1.2	9.7	2.6	3.6	2.5	23.5	2.0	6.3	3.7	2.63
Infiltrating carcinomas	50	2.1	1.4	3.0	2.8	1.8	2.8	3.2	2.3	4.5	4.2	1.08
	80	0.7	1.6	0.3	3.2	2.5	3.2	11.2	2.4	5.4	4.8	1.31
	104	8.1	1.5	6.8	3.0	2.4	3.0	10.6	2.3	6.8	4.5	1.17
	128	8.1	1.5	7.6	3.0	2.8	3.0	17.4	2.3	6.7	4.5	1.09
Standardised												
All tumours	50	12.1	1.8	13.1	3.0	5.1	3.6	10.4	3.2	8.5	5.1	0.06
	80	17.1	1.5	18.0	3.1	5.1	3.1	30.4	3.3	10.6	4.6	11.58
	104	21.1	1.9	18.8	4.0	4.7	4.0	37.8	3.7	10.1	5.9	10.39
	128	21.0	1.9	19.1	3.8	5.0	3.8	38.4	3.8	10.5	4.7	9.80
All carcinomas	50	3.5	1.5	3.2	3.1	1.2	3.1	4.3	2.4	4.7	4.6	0.52
	80	9.0	1.7	7.6	3.5	5.1	3.5	15.2	2.4	6.1	5.2	0.35
	104	13.0	1.5	12.4	3.0	4.5	3.0	24.3	2.7	9.1	4.5	5.36
	128	14.7	1.7	11.8	3.4	5.3	3.4	25.8	2.8	8.8	5.1	4.55
Infiltrating carcinomas	50	2.1	1.4	3.1	2.8	1.8	2.8	3.1	2.3	4.5	4.2	1.08
	80	7.1	1.7	7.1	3.4	2.9	3.4	11.6	2.4	5.8	5.1	0.83
	104	10.5	1.8	8.2	3.6	4.7	3.6	18.7	2.5	9.0	5.3	2.12
	128	11.2	1.9	10.1	3.7	5.0	3.7	19.1	2.5	9.0	5.5	1.58
Log (R) analysis												
Unstandardised												
All tumours	80	0.81	0.10	0.85	0.20	0.15	0.20	3.75	1.28	0.40	0.30	4.6
	128	0.03	0.10	0.51	0.18	0.13	0.18	3.10	1.36	0.28	0.28	1.7
Standardised												
All tumours	128	0.85	0.10	0.69	0.20	0.04	0.21	3.25	1.16	0.36	0.30	6.2
C.L. denotes half width of Confidence Limit P = 0.95 2 equal tails.												

C.L. denotes half width of Confidence Limit P = 0.05 2 equal tails.

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Since the linear relationship between the logarithm of dose and the response is then of great importance in interpretation of the results, an entirely different analysis, based upon the tumorigenic force, was done to check it.

## ANALYSIS OF TUMORIGENIC FORCE

Individual periods must be combined for this purpose in order to reduce the random variations, and a method of fitting standard statistical distributions to the tumorigenic force was used. This method is a generalisation of that used by Lea (1945) and approximated by Blanding *et al.* (1951) and it fits a lognormal distribution (with mean log tumour-induction time  $m$ , standard deviation  $s$ ) to the tumorigenic force, using the method of maximum likelihood. The generalisation allows a proportion  $c$  of the animals to remain tumourless indefinitely. This method was devised by Boag (1948, 1949) for examination of radiotherapy success in human cancer. Assuming that the distribution over time of the tumorigenic force in a given group of animals has either normal or lognormal form, it is then possible to write down the likelihood function for the observed data:

$$L = K \dots \sum_{i \in \text{Group 1}} \ln \left( \frac{1-c}{s} \right) \frac{1}{t_i} \div \sum_{i \in \text{Group 2}} \ln (c \div (1-c) \eta)$$

in which

$$\eta = \frac{1}{s} \left( \frac{t_i}{m} \right) \quad \text{or} \quad \ln(\eta) = \frac{\ln(t_i)}{s} - \frac{\ln(m)}{s}$$

for the two alternative forms

$$\frac{1}{2\pi} e^{-0.5x^2} \quad \text{and} \quad \eta = \int_x^\infty z dz$$

where  $L$  is the log likelihood,  $t$  the time,  $m$  the mean of the distribution,  $s$  its standard deviation and  $c$  the proportion of animals which would never develop tumours in the absence of natural mortality.

The summation over Group 1 covers all animals which develop tumours (tumour developing at time  $t$ ) and that over Group 2 all animals which die tumourless (at time  $t$ ).

The values of  $s$ ,  $c$  which maximise  $L$  can be found either by equating partial differential coefficients to zero in the usual way (as was done by Boag) or by using a maximum likelihood procedure such as Nelder's simplex method (Nelder and Mead, 1965). Computer program written in Algol are available for the latter technique.

The assumptions made about the form of the distribution of tumour incidence can then be rigorously tested by  $\chi^2$  goodness of fit tests. The distribution parameters will contain the information about difference between treatments, and analyses can be done on them.

This model has two great advantages over methods using rates. The distribution of tumours over time is defined as a distribution of tumorigenic force, which makes it possible to eliminate from the equations the mortality of the various groups of animals, and get satisfactory comparisons even of toxic treatments. This is, of course, the object of age standardised rates, but the fitted model over-

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comes the great disadvantage of age standardisation: that in treatments with excess mortality a single tumour may, in the standardised results, be weighed up to 4 or 5 and in a treatment with less than normal mortality it may count only 0.2 or 0.3. The maximum likelihood equations give each observation the weight needed to maximise the precision of the estimate; no difficulties are encountered when comparing treatment responses with different treatment-associated mortality rates nor any difficulties associated with selecting arbitrary time segments in the experiments.

Goodness of fit tests were done on the lognormal distributions fitted, and the values of  $\chi^2$  are shown in Table X. In Room 2 there are four significant values, but inspection of the data shows that all four are local anomalies between weeks 65 and 75, which are due to the change mentioned above in the standards of reporting illness and tumours. Since these results were so local it was not thought worthwhile to censor or attempt to smooth the data to eliminate them. One similar, though smaller anomaly was found in one treatment of Room 3. To give a more sensitive test of the fit of the model  $\chi^2$  tests were done on the sum of all treatments in each animal room. These were all very significant but the general picture was the same, with large local anomalies. The deviations in the four animal rooms were quite different, and a test on all four rooms taken together though still significant, showed smaller individual  $\chi^2$ 's. As a further check that these anomalies were local, and not due to a badly fitting model, normal distributions of tumorigenic force were fitted: and both individual treatment  $\chi^2$  tests and an overall  $\chi^2$  test were done. The results of these tests were almost identical with those of the lognormal tests, helping to confirm the local variation hypothesis.

Analyses of the normal distributions were done, but it was found that the standard deviation of the distributions increased with the means: and the patterns of variation between doses and treatments were less clear than those shown by the lognormal distributions, so the normal distribution results are not given in detail.

TABLE X.—Goodness of Fit Lognormal Model Tumours

Treatment		Room 1		Room 2		Room 3		Room 4	
No.		df	$\chi^2$	df	$\chi^2$	df	$\chi^2$	df	$\chi^2$
1	Control	1	0.02	2	0.02	1	0.25	2	0.50
2	Control acc.	1	0.00	1	0.38	1	0.06	1	0.35
3	Neutral low	3	2.48	3	2.33	2	0.67	4	1.10
4	Neutral med.	4	3.41	5	2.33	6	5.38	6	4.79
5	Neutral high	8	12.65	9	25.01	9	8.40	8	5.60
6	Stored low	1	1.01	3	1.79	3	4.28	2	0.10
7	Stored med.	6	7.32	7	14.51	4	2.98	4	1.57
8	Stored high	12	12.99	11	17.01	12	23.92	12	10.99
9	24-hour low	5	1.28	7	4.66	6	5.88	4	1.00
10	24-hour med.	11	18.18	11	35.19	9	9.23	8	4.74
11	24-hour high	13	8.47	12	11.42	11	14.73	10	3.05

Examples of Anomalies

Room 2 Treatment 10			Room 3 Treatment 5		
Weeks	Obs.	Exp.	Weeks	Obs.	Exp.
64-67	2	4.5	56-59	6	4.2
68-71	5	4.6	60-63	1	4.0
72-75	0	3.7	64-67	9	3.9
76-79	11	3.2	68-71	3	3.4
80-83	3	2.5			

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TABLE XI. *Fitted Distributions Means of Four Roanet-Lognormal Distributions*

Treatment and dose level	Tumours			Carcinomas			Infiltrating carcinomas		
	m	s	c	m	s	c	m	s	c
Controls, untreated .	5.302	0.517	0.438	5.745	0.304	0.007	5.975	0.332	0.744
Controls, Acetone .	5.451	0.499	0.485	5.995	0.351	0.747	5.995	0.351	0.747
Neutral fraction, low .	5.251	0.527	0.307	5.038	0.201	0.362	5.584	0.431	0.551
Neutral fraction, med. .	4.664	0.309	0.226	4.896	0.284	0.310	5.211	0.385	0.408
Neutral fraction, high .	4.530	0.345	0.165	4.729	0.272	0.259	4.822	0.300	0.289
Stored condensate, low .	4.952	0.383	0.316	5.121	0.277	0.411	5.594	0.337	0.587
Stored condensate, med. .	4.766	0.397	0.240	4.895	0.295	0.326	5.167	0.388	0.401
Stored condensate, high .	4.409	0.462	0.089	4.575	0.264	0.196	4.647	0.272	0.223
24 hr. condensate, low .	4.788	0.445	0.240	4.908	0.353	0.327	5.228	0.401	0.390
24 hr. condensate, med. .	4.490	0.397	0.130	4.660	0.257	0.237	4.831	0.325	0.280
24 hr. condensate, high .	4.396	0.444	0.086	4.607	0.231	0.217	4.731	0.263	0.269

m = mean tumour induction time.

s = standard deviation of tumour induction time.

c = proportion which would never develop tumours.

m and s are expressed in terms of  $\log_{10}$  (weeks) and c in terms of proportion.

CIGARETTE SMOKE CONDENSATE

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Table XI gives the means over four rooms of the three lognormal distribution parameters. The same analysis of variance as for rates, but without transformation, was done on the three parameters. It was found that the standard deviations did not significantly change from one treatment to another or between dose levels. In Tables XII and XIII have been extracted the mean tumour-induction times for the three dose rates and for the three treatments.

TABLE XII.—Means of Lognormal Distributions

	Tumours		Carcinomas		Infiltrating carcinomas	
	log	weeks	log	weeks	log	weeks
Low dose	4.913	136	5.042	155	5.469	235
Medium dose	4.849	103	4.817	124	5.066	159
High dose	4.445	85	4.637	102	4.734	114
C.L.S. ( $p = 0.05$ )	0.053	—	0.090	—	0.176	—
Z-test effect	0.234	—	0.203	—	0.367	—
C.L.S. ( $p$ vs 0.05)	0.038	—	0.064	—	0.124	—

TABLE XIII.—Means of Lognormal Distributions

	Tumours		Carcinomas		Infiltrating carcinomas	
	log	weeks	log	weeks	log	weeks
Neutral fraction	4.731	114	4.888	137	5.206	156
Stored condensate	4.769	110	4.863	135	5.132	152
24-hr. condensate	4.558	95	4.745	104	4.930	139
C.L.S. ( $p = 0.05$ )	0.053	—	0.090	—	0.176	—
Mean	4.666	—	4.832	—	5.080	—

fall for mean tumour-induction time as the dose increases is not significantly different from a linear change (in the log time); and the differences in the rate of change between the three tumour responses are not significant, the average fall for a doubled dose being  $0.235 \pm 0.031$  or about 27%.

The three types of condensate show the same pattern in all three responses with stored condensate having a mean a little below that of neutral fraction (0.024 or 3%) and 24-hour old condensate appreciably lower (0.177 or 19%). The means differ significantly for the three tumour responses in the order all tumours (lowest), carcinomas, and infiltrating carcinomas (highest). The stored condensate anomaly and the similarity of 24-hour condensate high and 24-hour condensate medium dose rates can still be seen but they are not significant. For the proportion of animals which never develop tumours (c) the difference between treatments are consistent, showing a fall of about 1% between neutral fraction and stored condensate and about 7% between neutral fraction and 24-hour condensate. The stored condensate anomaly is significant only in the analysis of c for all tumours.

## MEASUREMENT OF TUMORIGENICITY

There are many response scales which can be used to measure tumorigenicity for example tumour rates at different times in the experiment. These scales will give different ratios for the relative response of two substances as shown in Table

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IX. In many cases however the response ratio for two substances varies from scale in the same way as the response to different doses of a single substance. A dose level of one substance can be found which would give the same response in every scale as a known dose of a second substance. Relative tumorigenicity can then be defined as a dose ratio rather than a response ratio, in a way analogous to LD<sub>50</sub> in toxicity trials. Toxicity trials are however simpler than carcinogen assays because they usually last a relatively short time, while the carcinogen assays must cover nearly the whole life span of the test animals, and it must be shown that a relative tumorigenicity does not change with time during the trial.

To fulfil this requirement the response-time curves for different substances must have the same shape (though they may vary both in magnitude of response and in time of appearance of tumours). The condition for making valid comparisons of tumorigenicity may be defined:

The relative tumorigenicity of two substances is  $r$  if, and only if, a dose  $D$  of one produces the same distribution of tumour incidence as a dose  $rD$  of the second, when these doses are administered in the same way to random samples of a single population of animals.

This definition implies that in many cases it will not be possible to define a relative tumorigenicity because the shapes of distributions of incidence are not sufficiently similar.

In the present work this type of comparison was justified by showing that the time-response curves for individual treatments all fitted the lognormal distributions, so that all the information could be concentrated into the three parameters  $m$ ,  $s$  and  $c$ , which proved to be highly correlated over the population of 44 groups of mice and three responses. Either  $m$  or  $c$  could therefore be considered as a single response parameter permitting a valid measurement of "relative tumorigenicity" to be made for the three treatments.

As a check on the fit of this model, graphs of the tumorigenic force  $T$  were prepared. All showed that  $T$  increased with time; but when straight lines were fitted, allowing for the decreasing weight of the observations as the number of animals falls, it was found that the slopes were proportional to the mean, so that the condition was fulfilled. The similarity is shown by Fig. 7, in which the uncorrected number of tumour-bearing animals for neutral fraction high dose has been matched by interpolating between 24-hour condensate medium and low doses.

The analysis of variance of the tumour-induction time  $m$  or proportion of animals which never develop tumours  $c$  can then be used to interpolate between the responses for different dose levels to get relative carcinogenicities for different smoke condensates and Fisher's method can be used to obtain fiducial limits for the ratios (Fisher, 1946). No difference was found between the results (Table V above) obtained with either parameter or those for all tumours, carcinomas and infiltrating carcinomas and the mean ratios of carcinogenicity are given in Table IV. This similarity of the curves obtained for the different condensates and types of tumour suggests that the mechanism of tumour production is the same for all three types of condensate.

The use of ratios obtained using the lognormal model depends of course on the assumptions:

- (a) The model is realistic and since the tests of fit are not unsatisfactory the parameters obtained do in fact represent the data.

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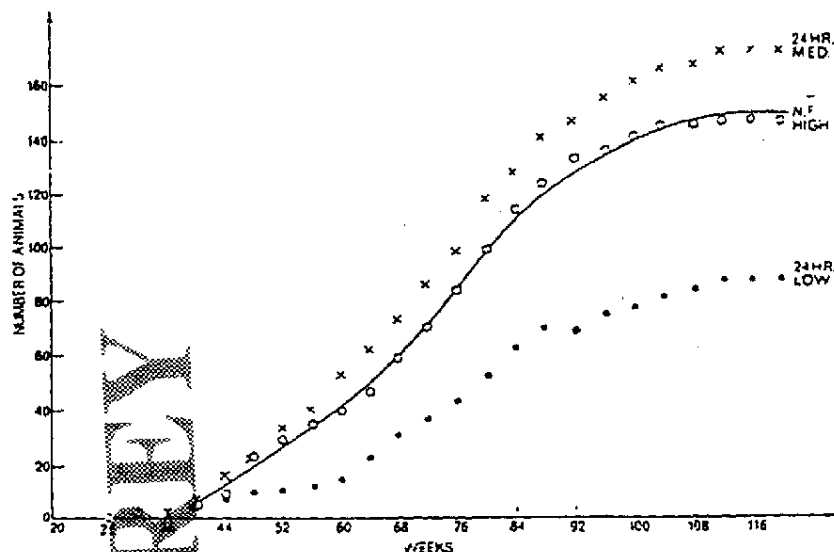


FIG. 7.—Similarity of shape for Time Response curves. The circles show the Neutral Fraction High Dose results for one room. The solid line is a linear interpolation between 24 hour condensation medium and low curves.

(b) The division of information shown by the analysis of variance, with dose response relationship in  $m$  and  $c$  and random variations in  $s$  has not in fact destroyed relevant information on dose response relationships. If either of these assumptions has to be rejected the results must be expressed using incidence rate measures of response, as in the right-hand side of Table IV.

#### CONCLUSIONS

Two important conclusions of interest to statisticians can be drawn from this experiment.

1. In tests of weak carcinogens where it is essential to use the maximum possible dose, and to continue treatment until death, there will often be differences in mortality between dose levels and treatments. It is important to allow for this in the analysis of the results, if it is possible to make the basic assumptions so that correction is possible.
2. The lognormal distribution of tumorigenic force, with some animals never developing tumours, fits the data from this experiment. It provides a simpler picture than the other analyses which have been done.

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